GRAM genes and abscisic acid (ABA) metabolism in the reproductive development of *Arabidopsis thaliana*

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KEVIN NEAL BARON

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ABBREVIATIONS

AAO, abscisic aldehyde oxidase

ABA, abscisic acid

ABA-GE, abscisic acid glucose ester

aba1, ABA deficient 1

abi1, ABA-insensitive 1

ABC transporters, ATP-binding cassette transporters

ABF/AREB, ABRE Binding Factors/Abscisic Acid Responsive Elements Binding Factor

ABRE, abscisic acid response element

AP2/ERF, APETALA2/ETHYLENE RESPONSE FACTOR

ATG, "autophagy-related" gene

Atg, "autophagy-related" protein

CRT/DRE, C-repeat/dehydration response element

DAG, diacylglycerol kinase

GRAM, **G**lucosyltransferases, **Ra**b-like GTPase activators and **M**yotubularins

IP_3, inositol trisphosphate

IP_6, inositol hexaphosphate

MDC, monodansylcadaverine

MTM/MTMR, Myotubularin/Myotubularin-Related

MYC/MYB, myeloblastosis/myelocytomastosis

NAC, NAM, ATAF1,2 CUC2

NCED, nine-*cis*-epoxycarotenoid dioxygenase

PA, phosphatidic acid

PLC, phospholipase C

PLC, phospholipase D

PKC, protein kinase C

PP2Cs, protein phosphatase 2Cs

PtdIns, phosphatidylinositol

PYR/PYL/RCAR, pyrabactin resistance, PYR1-like, regulatory components of ABA receptor

ROS, reactive oxygen species

RT-qPCR, real time quantitative polymerase chain reaction

SnRK, SNF1-related protein kinases

WRKY, ‘worky’ transcription factor family

ABSTRACT

Baron Kevin Neal. Ph.D., The University of Manitoba, April, 2013. **GRAM genes and abscisic acid (ABA) metabolism in the reproductive development of *Arabidopsis thaliana***

Supervisor: Dr. Claudio Stasolla (Plant Science)

Co-Supervisor: Dr. Dana Schroeder (Biological Sciences)

Abscisic acid (ABA) is a key plant hormone regulating agronomically important processes including seed maturation and dormancy, stomatal opening and closure, along with the transcriptional and physiological response of plants to abiotic and biotic stresses. The current study sought to functionally characterize members of an ABA-responsive gene family encoding GRAM (**G**lucosyltransferases, **Ra**b-like GTPase activators and **M**yotubularins) domain proteins in *Arabidopsis thaliana*. Utilizing reverse genetics loss- and gain-of-function lines associated with ***GE****M-****R****ELATED* ***5*** (*GER5*) were obtained, which displayed several defects in reproductive development. Gene expression profiling, RNA *in situ* hybridization and immunohistochemical techniques were utilized to evaluate *GER5* and two closely related GRAM genes, ***GE****M-****R****ELATED* ***1*** (***GER1)*** and ***G****LABRA2* ***E****XPRESSION* ***M****ODULATOR* (***GEM***) in reproductive structures. Microarray profiling of seeds from *ger5-2* mutants and wild-type plants revealed transcriptional changes in carbohydrate metabolism, hormone signaling and catabolic processes accompanied seed development defects of *ger5-2* mutants. Seed germination assays further revealed *ger5-2* mutants exhibited reduced sensitivity to ABA.

In assessing *GER5, GER1* and *GEM* as putative ABA-response genes, a second study evaluated the expression of GRAM, AuTophaGy-related (*ATG*), and ABA-response genes in source and sink organs exposed to abiotic stress or within mutant backgrounds deficient in sugar signaling. Monodansylcadaverine (MDC) staining was also utilized to localize autophagosomes or autophagic bodies within vegetative or reproductive organs during plant development, or in response to carbon starvation or abiotic stress.

In a third study transcriptional differences in ABA metabolism, transport and homeostasis were examined within reproductive organs (cauline leaves, inflorescence meristem, developing siliques) exposed to cold and heat stress. This study revealed reproductive organs are characterized by unique patterns of ABA metabolism which differ from tissues typically associated with classical ABA responses. Together, these studies indicate *GER5*, an uncharacterized ABA-responsive GRAM domain gene, plays a novel role in the reproductive development of plants and that ABA metabolism and signaling are uniquely regulated in reproductive organs.

FOREWORD

This thesis follows the manuscript style outlined by the Department of Plant Science, University of Manitoba. The manuscripts follow the style recommended by Planta. This thesis is presented as three manuscripts, each containing an abstract, introduction, materials and methods, results and discussion section. For each manuscript, supplemental figures and tables are placed immediately following the body of the manuscript. A literature review precedes the manuscripts and general discussion follows the manuscripts.

1.0 INTRODUCTION

Abscisic acid (ABA) is a major phytohormone that functions as a stress-related signaling molecule mediating plant adaptation to environmental conditions such as drought, salinity, and hypoxia in addition to low and high temperature extremes (Finkelstein et al. 2002). ABA also plays a critical role in water relations of plants. During drought stress ABA accumulation in leaves triggers a signaling cascade and physiological steps that induce stomatal closure and inhibit stomatal opening, thus leading to reductions in transpirational water loss (Kwak et al. 2003; Wilkinson and Davies 2010). ABA also regulates aquaporins, which mediate the cell-to-cell movement of water, and synthesis of proteins (e.g. dehydrins, LEA) that protect vegetative tissues undergoing cellular dehydration (Finkelstein et al. 2002). During plant growth and development, ABA directs the synthesis and accumulation of seed storage proteins and lipids in addition to promoting dessication tolerance and dormancy of seeds. A classical role for ABA in plant physiology surrounds antagonistic interactions with gibberellins, that regulate seed dormancy and germination (Koornneef et al. 1982; Nambara and Marion-Poll 2005).

At a cellular and molecular level, forward and reverse genetic studies have identified numerous loci which participate in ABA metabolism and signaling (Finkelstein et al. 2002; Cutler et al. 2010). Molecular studies of this nature often occur in model species such as *Arabidopsis thaliana* or *Oryza sativa*, and tend to focus on "classical" ABA responses described above. Genetic studies in *A. thaliana* also revealed a prominent role for ABA in sugar signaling pathways (Arenas-Huertero et al. 2000; Huang et al. 2008). In recent years, considerable progress has been made toward

characterizing upstream components of the ABA signal transduction pathway including the isolation of ABA receptors and their functional relationship to positive and negative regulators of ABA-dependent gene expression programs (Cutler et al. 2010). In spite of these advances our understanding of ABA signaling and physiology remains incomplete and novel roles for ABA in plant growth, development and stress response(s) continue to emerge (Yang et al. 2006a; Ton et al. 2009; Zhang et al. 2010). In addition, many putative ABA- and stress-responsive genes remain to be functionally characterized.

In plants, relatively little is known regarding the function of genes encoding GRAM (**G**lucosyltransferases, **Ra**b-like GTPase activators and **M**yotubularins) domain proteins, although members appear responsive to abscisic acid (ABA), nutrient deprivation or environmental stress (Liu et al. 1999; Hoth et al. 2002; Riccardi et al. 2004; Yazaki et al. 2004; Lee et al. 2005; Jiang et al. 2008; Choi and Hwang 2011). In the present study, characterization efforts initially focused upon *GER5* (***GE****M-****R****ELATED* **5**/At5g13200), an uncharacterized member of the GRAM domain gene family in *A. thaliana* and ortholog of barley (*Hordeum vulgare*) *ABA45*, the first GRAM domain sequence identified in plants (Liu et al. 1999). Based upon phenotypes which emerged in mutant and transgenic lines, characterization efforts expanded to include two closely related members of the GRAM gene family in *A. thaliana*, *GEM* (***G****LABRA2* ***E****XPRESSION* ***M****ODULATOR*/At2g22475) and *GER1* (***GE****R-****R****ELATED* ***1***/At1g28200).

In eukaryotic organisms, several genes encoding GRAM domain proteins regulate autophagy, a non-selective catabolic degradation process responsible for recycling organelles or cytosolic macromolecules during carbon starvation (Oku et al. 2003; Vergne et al. 2009). Increasing evidence suggests ABA functions as a plant specific

regulator of autophagy. As phenotypes associated with loss- or gain-of-*GER5* function closely parallel reproductive phenotypes associated with ABA-responsive genes (*HVA22/AtHVA22*) known to participate in autophagy (Chen et al. 2009), a second study sought to determine how abiotic stress or defects in sugar signaling influenced the transcriptional and morphological response of autophagy in reproductive organs.

The majority of studies assessing ABA biosynthesis and catabolism in *A. thaliana* have focused on seed dormancy and germination, seedling development or the response of vegetative tissues to abiotic stress (Nambara and Marion-Poll 2005). The final study examined whether rapidly growing, undifferentiated inflorescence meristems or developing siliques were characterized by unique features of ABA metabolism, transport or homeostasis relative to fully expanded cauline leaves. In addition, whether ABA metabolism and transport are differentially regulated in these same reproductive organs exposed to abiotic stress.

2.0 LITERATURE REVIEW

2.1 Abscisic acid (ABA)

2.1.1 The phytohormone abscisic acid (ABA)

The phytohormone abscisic acid (ABA) was originally isolated from plants in the early 1960s as a compound capable of accelerating the senescence of cotton (*Gossypium* spp) fruit or inducing dormancy in the leaves of sycamore (*Acer pseudoplatanus* L) (Ohkuma et al. 1963; Conforth et al. 1965). Isolated and named as separate compounds according to their physiological roles, a subsequent comparison of spectral properties revealed that 'abscisin II' and 'dormin' were in fact identical and led to renaming of the compound as abscisic acid (ABA). By the late 1960s it became apparent that ABA was capable of causing stomata to close and that inhibitory stress compounds and ABA accumulate rapidly in leaves during wilting stress (Milborrow 2001).

The production of synthetic ABA compounds and their subsequent utilization in physiological bioassays revealed the action of several growth-promoting compounds (e.g. gibberellins) was countered by the action or activity of ABA (Koornneef et al. 1998). Synthetic ABA compounds were also crucial for assays which enabled the identification of mutants deficient in ABA biosynthesis and signaling (Koornneef et al. 1998). To date a majority of ABA biosynthesis and signaling mutants have been identified in the model plant *A. thaliana*, although ABA mutants have been isolated in tobacco (*Nicotiana tabacum*), corn (*Zea mays*), pea (*Pisum sativum*) and tomato (*Lycopersicon esculentum*). Apart from *A. thaliana* mutants described in subsequent sections the most well characterized ABA biosynthesis and signaling mutants are the wilty mutants of tomato

(*flacca*, *sitiens*, *notabilis*) and the *viviparous* mutants of maize (*Zea mays*) (Taylor and Tarr 1984; Tan et al. 1997).

At a physiological level the plant hormone abscisic acid (ABA) mediates an array of agronomically important stress and developmental responses including seed storage protein synthesis, promotion of seed dessication tolerance and dormancy, leaf water relations and tolerance to environmental stress such as cold, drought and salinity (Leung and Giraudat 1998; Finkelstein et al. 2002). More recently classic physiological roles for ABA have been amended to include pathogen response, root development and photoprotection (De Smet et al. 2006; Yang et al. 2006a; Ton et al. 2009). ABA responses are also frequently partitioned into fast and slow responses (Finkelstein et al. 2002). For example, stomatal responses occur relatively fast (seconds to minutes) and recruit signaling intermediates and ion channels to regulate stomatal opening and closure. In comparison, ABA-mediated changes in gene expression that follow environmental stress occur over longer time frames (hours to days), but frequently recruit the same signaling intermediates operative during fast ABA responses (Zeevaart and Creelman 1988; Finkelstein et al. 2002).

At a molecular level, ABA signaling appears particularly complex in part due to a highly branched signaling pathway with multiple redundant ABA perception and signaling mechanisms (Finkelstein et al. 2002; Cutler et al. 2010). In spite of this complexity, molecular genetic approaches have identified a large number of genes (over 100 loci) and secondary messenger molecules (e.g. Ca^{2+}, reactive oxygen species, phospholipids) involved in ABA signaling (Finkelstein et al. 2002). Recent molecular advances in the ABA field include the isolation of a genetically redundant family of

nucleo-cytoplasmic ABA receptors (PYR/PYL/RCAR) which, upon binding ABA, physically interact with and inhibit type 2C protein phosphatases (PP2Cs) that act as upstream negative regulators of the ABA signaling pathway (Cutler et al. 2010). Additional plastid (CHLH) and plasma membrane (GTG) localized ABA receptors have also been identified (Shen et al. 2006; Pandey et al. 2009), although their respective roles in the overall topology of the ABA signaling network are just beginning to emerge (Cutler et al. 2010; Shang et al. 2010). Studies of ABA metabolism and transport have advanced through the isolation of organelle-specific β-glucosidases (AtBG1, AtBG2) capable of hydrolyzing abscisic acid glucosyl ester (ABA-GE) (Lee et al. 2006; Xu et al. 2012) and multiple proteins (AtABCG22, AtABCG25, AtABCG40, AIT) which facilitate intercellular transport of ABA (Kang et al. 2010; Kuromori et al. 2010; 2011; Kanno et al. 2012).

In the following sections general aspects of ABA metabolism, signaling and physiology are presented with specific attention given to the model plant *A. thaliana* in addition to the role of ABA in the reproductive development of plants. Recent studies and reviews conducted across monocot and dicot crop species have reinforced the importance of ABA metabolism and signaling, and integration with sugar and stress signaling pathways, as key mediators of senescence and early reproductive processes in economically important crop species (Liu et al. 2005; Oliver et al. 2007; Barnabás et al. 2008; Thakur et al. 2010; Zhu et al. 2010). However, in *A. thaliana*, relative to classical ABA responses (e.g. stomatal closure, seed dormancy, accumulation of seed storage proteins) early stages of reproductive development corresponding to male and/or female gametophyte development have received considerably less attention with respect to ABA

metabolism and signaling. The following sections focus prominently on *A. thaliana* but it is important to emphasize that molecular components of ABA metabolism and signaling are generally conserved across higher plant species with many functions conserved in lower plants (Nambara and Marion-Poll 2005). The similarities between mutant phenotypes of ABA biosynthesis and signaling mutants from diverse species (*Nicotiana plumbaginifolia*, *Zea mays*, *Lycopersicon esculentum*, *Pisum sativum)* lend further support to the notion that ABA effects observed in *A. thaliana* can be extrapolated to other plant species.

2.1.2 Abscisic acid metabolism and transport in plants

At any given time, ABA levels in plant cells and organs are modified by the combined action of biosynthesis, catabolism and conjugation reactions, with long range transport of ABA and ABA conjugates contributing to the ABA metabolism of an individual plant cell. Metabolic steps upstream and downstream of ABA contribute equally to fine-tuning endogenous ABA levels, and to date most genes involved in ABA biosynthesis have been cloned or identified through mutant analysis (Nambara and Marion-Poll 2005; North et al. 2007). While ABA is broadly considered to function as a 'growth inhibitor' early reviews of ABA metabolism noted that ABA biosynthesis mutants (which contain lower levels of a suspected growth inhibitor) actually exhibit reduced growth rates which can be rescued by supplementing biosynthesis mutants with exogenous ABA (Zeevaart and Creelman 1988). These observations suggest that characterizing ABA as a growth inhibitor may not accurately reflect the role of this hormone in plant growth and development.

Early studies of ABA metabolism focused on isolating, purifying and quantifying ABA in plant extracts, or utilizing stable isotopes to track the conversion of C_{15} and C_{40} (carotenoid) precursors into ABA (Zeevaart and Creelman 1988; Milborrow 2001). Radioactive ABA compounds also enabled researchers to track the movement and catabolism of ABA within and between plant organs. From these early physiological and biochemical studies it became apparent that the biosynthesis, catabolism and turnover of ABA and ABA-GE differ vastly when turgid leaves were contrasted with stressed leaves (Zeevaart and Boyer 1984).

While generally accepted that environmental stress stimulates ABA biosynthesis and an increase in cellular ABA content, it is often underappreciated how ABA levels are coordinately regulated in response to transient or sustained periods of stress (Nambara and Marion-Poll 2005). For example, after several hours of imposed stress elevated levels of endogenous ABA are often accompanied by an accumulation of ABA catabolites such as phaseic acid (PA) and dihydrophaseic acid (DPA), suggesting maintenance of elevated ABA levels in stressed tissue is attributed to a regulated balance between biosynthesis and catabolism (Zeevaart and Creelman 1988; Nambara and Marion-Poll 2005). Early metabolic studies also revealed leaf age exerts a significant influence upon ABA metabolism. Young leaves generally have the highest levels of ABA, but a diminished capacity to synthesize or catabolize this hormone, suggesting that most ABA present in young leaves or shoot meristems is imported from older leaves (Zeevaart and Boyer 1984; Zeevaart and Creelman 1988). Similar metabolic studies demonstrated endogenous ABA and exogenous ABA are metabolized in different manners. In turgid leaves, endogenous ABA may be sequestered away from catabolic

enzymes in certain organelles (chloroplast) whereas exogenous ABA is rapidly converted in the cytoplasm by catabolic enzymes (Zeevaart and Creelman 1988). A subset of studies further revealed an intense focus on total ABA content of tissues often overlooks subtle changes in compartmentation (apoplasm, chloroplast, vacuole, cytoplasm) following environmental stress or the transition from light to dark conditions (Zeevaart and Creelman 1988; Milborrow 2001).

The identification of ABA biosynthesis mutants and their subsequent utilization in physiological and biochemical studies formed the framework of molecular studies seeking to identify and clone loci involved in ABA biosynthesis (Koornneef et al. 1998). *aba1, aba2, aba3* and recent *aba4* biosynthetic mutants of *A. thaliana* were all isolated based upon genetic screens to identify mutants capable of germinating in the presence of the gibberellin (GA) biosynthesis inhibitor paclobutrazol (Koornneef et al. 1982; Léon-Kloosterziel et al. 1996; North et al. 2007). A key control point in ABA metabolism include the zeaxanthin epoxidase (*ZEP*) gene, [corresponding to the *ABA1* locus (*aba1* mutants) in *A. thaliana* and *ABA2* locus (*aba2* mutants) in *Nicotiana plumbaginifolia*], responsible for two-step synthesis of all-*trans*-violaxanthin from zeaxanthin with antheraxanthin serving as an intermediate (Fig. 2.1). In turn all-*trans*-violaxanthin is converted to either 9'-*cis*-violaxanthin or 9'*cis*-neoxanthin. The recently identified *ABA4* locus (*aba4* mutants), encoding a chloroplast localized protein, mediates the *de novo* synthesis of 9'*cis*-neoxanthin (North et al. 2007). Once formed, oxidative cleavage of 9'-*cis*-violaxanthin or 9'*cis*-neoxanthin is executed by a family of 9'-*cis*-epoxy carotenoid dioxygenase (NCED) proteins which co-localize with C_{40} substrates in chloroplasts (Fig. 2.1). Cleavage of C_{40} carotenoid precursors (violaxanthin and neoxanthin) by NCEDs

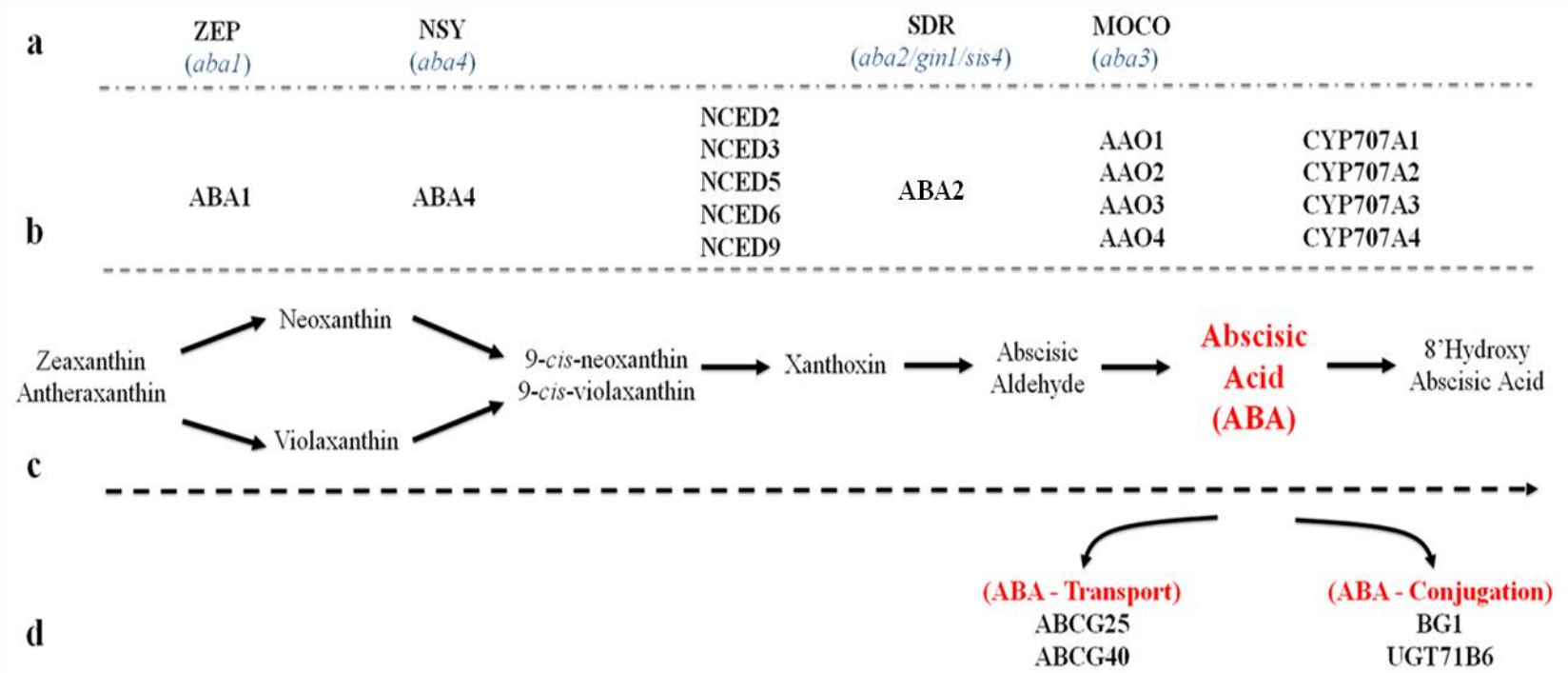

Fig. 2.1 Schematic representation of ABA metabolic pathway. **a** enzymes associated with key ABA biosynthesis mutants (ZEP = zeaxanthin epoxidase, NSY = neoxanthin synthase, SDR = short chain alcohol dehydrogenase/reductase, MOCO = molybdenum cofactor sulfurase). ABA biosynthesis mutants associated with defects are displayed in blue. **b** *Arabidopsis thaliana* loci associated with key steps in ABA biosynthesis and catabolism. In several cases multi-gene families mediate the conversion of pathway intermediates. **c** Precursors involved in the formation of physiologically active ABA or the conversion of ABA to ABA catabolites (e.g. 8'hydroxy ABA). **d** Loci associated with interconversion of ABA and ABA-glucose ester, loci associated with intracellular or extracellular transport of ABA. The figure above has been based upon Nambara and Marion-Poll (2005) and Umezawa et al. (2010). Permission has been obtained from the publisher/copyright holder to incorporate these figures in the thesis.

generates xanthoxin, a C_{15} compound and precursor of ABA. This particular step is considered rate-limiting for ABA biosynthesis in plants (Nambara and Marion-Poll 2005). While some controversy surrounds the final subcellular location of ABA biosynthesis, the *ABA2* locus (*aba2* mutant), corresponding to a short-chain alcohol dehydrogenase/reductase (SDR), is responsible for the oxidation of xanthoxin to abscisic aldehyde (Fig. 2.1) (Cheng et al. 2002; González-Guzmán et al. 2002). The final step in ABA biosynthesis involves the conversion of abscisic aldehyde to ABA and in *A. thaliana* is mediated by an abscisic aldehyde (AAO) gene family (*AAO1-AAO4*) (Seo et al. 2004). However, amongst members of this family AAO3 is considered the dominant enzyme responsible for ABA biosynthesis during seed development and drought stress (Koiwai et al. 2004; Seo et al. 2004). AAO enzymes additionally require sulfurylated molybdenum as a cofactor and in the case of *aba3* biosynthesis mutants ABA deficiency results from defective molybdenum cofactor sulferase (MOCO) activity (Xiong et al. 2001a).

In contrast to the use of forward genetics to identify early ABA biosynthesis mutants in model and crop species, the genes and enzymes involved in ABA catabolism have only recently been examined through reverse genetics approaches (Nambara and Marion-Poll 2005). Nonetheless, catabolic reactions appear to hold equivalent roles in regulating ABA levels throughout plant development and in response to environmental stimuli (Millar et al. 2006; Okamoto et al. 2009; Zhu et al. 2009). The ABA catabolic pathway is generally delineated by hydroxylation and conjugation pathways which convert ABA to physiologically inactive forms. Of the two catabolic pathways oxidation of ABA by ABA 8' hydroxylase is considered the major catabolic route in higher plants,

and in *A. thaliana* is mediated by four members of a gene family (*CYP707A1-A4*) encoding cytochrome P450 monooxygenases (Nambara and Marion-Poll 2005). Once formed 8' hydroxyl ABA spontaneously undergoes cyclization to form phaseic acid (PA) which in turn is catabolized to dihydrophaseic acid (DPA). 7' hydroxyl ABA and 9' hydroxyl ABA catabolites also exist in plants although little is currently known regarding these catabolic pathways (Nambara and Marion-Poll 2005).

ABA and hydroxylated catabolites can also be inactivated through conjugation with glucose (Nambara and Marion-Poll 2005). In plants, ABA-glucosyl ester (ABA-GE) is the most common conjugate identified and increased focus has been placed upon ABA-GE as a putative intermediate involved in inter-organ or root-to-shoot ABA transport (Sauter et al. 2002). Glucosyltransferases capable of conjugating ABA with UDP glucose have been identified in *Vigna angularis* (Adzuki bean) and *A. thaliana* although their respective roles in ABA metabolism remain somewhat obscure (Xu et al. 2002; Priest et al. 2006). ABA-GE and related conjugates were previously regarded as physiologically inactive catabolites that accumulate in plant vacuoles or the apoplastic space (Zeevaart and Creelman 1988). However, the isolation and characterization of an ER-localized β-glucosidase (AtBG1) enzyme in *A. thaliana* revealed plants were capable of rapidly hydrolyzing intracellular ABA-GE to increase active pools of extracellular ABA during dehydration stress (Lee et al. 2006). Xu et al. (2012) also recently identified AtBG2 as a vacuole-localized homolog of AtBG1 capable of hydrolyzing ABA-GE to ABA. Prior to the identification of AtBG1 and AtBG2 in *A. thaliana*, Dietz et al. (2000) also isolated an extracellular (apoplastic) β-glucosidase activity from barley leaves which liberated substantial quantities of free ABA from the apoplastic ABA-GE pool.

However, the extreme hydrophilic properties of ABA-GE which hamper passive transport across membranes led Sauter et al. (2002) to propose ATP-binding cassette (ABC) transporters may participate in active loading of ABA-GE within xylem parenchyma cells. While ABA-GE specific transporters remain to be identified in plants, separate groups have isolated several ABC transporters (AtABCG22, AtABCG25, AtABCG40) and a NRT/PTR transporter that function as ABA transporters in *A. thaliana* (Kang et al. 2010; Kuromori et al. 2010; 2011; Kanno et al. 2012).

2.1.3 Regulation of abscisic acid metabolism

Through forward and reverse genetic approaches plant biologists have identified a large suite of genes and gene families that participate in ABA metabolism and signaling (Koornneef et al. 1998; Nambara and Marion-Poll 2005). While early metabolic studies focused upon the movement or incorporation of radiolabelled precursors or intermediates into ABA or ABA catabolites (Zeevaart and Creelman 1988; Milborrow 2001), more recent molecular studies have examined the transcriptional activation or repression of biosynthetic (*ABA1, ABA2, NCED*) or catabolic (*CYP707A*) genes that modify endogenous ABA levels during development or in response to abiotic or biotic stresses (Nambara and Marion-Poll 2005). Molecular studies of this nature are typically restricted to model species such as *A. thaliana* or *O. sativa* and tend to focus upon classical physiological ABA responses such as seed development, seed dormancy and germination, or the response of vegetative tissues to dehydration and drought stress (Iuchi et al. 2001; Seo et al. 2004; Barrero et al. 2006; Millar et al. 2006).

Several ABA biosynthesis genes (*ABA1, ABA3, AAO3* and *NCED3*) are transcriptionally responsive to exogenous ABA or increases in intracellular ABA levels,

suggesting the entire ABA biosynthetic pathway may be subjective to a positive feed-forward mechanism that responds to cellular ABA levels (Xiong and Zhu 2003; Barrero et al. 2006). A number of stress-inducible ABA biosynthesis genes (*NCED3*, *ABA1*) harbour *cis*-elements associated with both ABA-dependent and ABA-independent signaling pathways, suggesting transcription factors associated with stress response pathways may be capable of activating genes (*NCED3*, *AAO3*, *ABA1*) involved in *de novo* ABA biosynthesis (Xiong and Zhu 2003; Barrero et al. 2006). In support of the notion that ABA-independent pathways activate the ABA biosynthetic pathway, Barrero et al. (2006) discovered that in severe ABA-deficient mutants (*aba1*, *aba2* and *aba3)* salt stress activated ABA biosynthesis, with *NCED3* emerging as the most likely target for ABA-independent activation.

In comparison, the *ABA2* biosynthesis gene, which is not transcriptionally responsive to ABA treatments, is specifically upregulated by glucose, but is not activated by drought or osmotic stress (Cheng et al. 2002; Gonzàlez-Guzmàn et al. 2002). *NCED3*, in contrast, is upregulated by drought and osmotic stress, but not glucose (Iuchi et al. 2001; Leon and Sheen 2003). However, more recent results indicate that two new *sugar insensitive* mutants (*sis7*, *sis10*) carry mutations in key ABA biosynthesis (*NCED3/SIS7*) and signaling (*ABI3/SIS10*) genes (Huang et al. 2008). RT-qPCR studies also determined (in germinating seeds as opposed to young seedlings), that glucose could increase transcript levels of several ABA biosynthesis genes (*ABA1*, *NCED3*, *ABA2*, *ABA3*) (Huang et al. 2008).

NCED genes are considered to encode key regulatory enzymes controlling ABA biosynthesis as their expression level tends to correlate with endogenous ABA content

(Nambara and Marion-Poll 2005). However, for some physiological processes ABA 8'hydroxylases serve as key determinants of endogenous ABA level. In *O. sativa*, accumulation of ABA during glucose-mediated inhibition of germination was found to correlate more with suppression of ABA catabolic genes (*OsABA8ox2* and *OsABA8ox3*) as opposed to upregulation of ABA biosynthesis genes (Zhu et al. 2009). In *A. thaliana*, ABA 8'-hydroxylase transcripts also respond to environmental stress (salinity, osmotic, dehydration) and exogenous applications of ABA (Kushiro et al. 2004; Umezawa et al. 2006).

However, the timing and intensity of ABA 8'-hydroxylase activity may initially not be matched by that of NCED enzymes, suggesting that during environmental stress ABA levels increase through the dynamic and coordinated action of *NCED* and *CYP707A* genes (Nambara and Marion-Poll 2005). During the dehydration/rehydration response of leaves or following subtle changes in atmospheric humidity, ABA 8'-hydroxylases function to reduce endogenous ABA to basal levels (Umezawa et al. 2006; Okamoto et al. 2009). Models have also emerged to explain how certain genes in the ABA biosynthetic pathway (*ABA1*, *AAO3* and *NCED3*) are subject to feed forward positive regulation by endogenous ABA under certain developmental or physiological contexts (Xiong and Zhu 2003; Barrero et al. 2006). In the absence of stress, exogenous applications of ABA or in transgenic plants engineered to overaccumulate ABA, the increased level of ABA would be accompanied by increased activity of ABA 8' hydroxylases and the appearance of ABA catabolites (Cutler and Krochko 1999).

A critical aspect of ABA metabolism and signaling surrounds the capacity for other phytohormones (gibberellins, brassinosteroids and ethylene) to regulate ABA

metabolism. Molecular studies have identified substantial reciprocal regulation of ABA metabolism during seed dormancy and germination or early seedling development (Seo et al. 2006; Cheng et al. 2009). The most recent example of reciprocal phytohormone-mediated regulation of ABA metabolism surrounds the antagonism between cytokinin and ABA in mediating osmotic stress signaling pathways linked to the function of a histidine kinase receptor ATHK1 (Tran et al. 2007; Wohlbach et al. 2008). During vegetative development and seed maturation, an osmotic stress pathway initiated by ATHK1 induces several ABA biosynthesis genes (*ABA1*, *ABA2* and *AAO3*) and coordinates the transcriptional activation of both ABA-dependent and ABA-independent signaling pathways (Tran et al. 2007; Wohlbach et al. 2008). Genetic studies further revealed cytokinin receptor histidine kinases (AHK2, AHK3 and CRE1) homologous to ATHK1 antagonized ATHK1 osmotic and ABA signaling pathways (Tran et al. 2007).

Apart from direct transcriptional regulation of genes involved in ABA biosynthesis and catabolism, RNA metabolism and post-translational modifications serve as control points in ABA metabolism. In the case of RNA metabolism, *sad1* (supersensitive to ABA and drought1) mutants of *A. thaliana*, defective in an Sm-like snRNP protein, display impaired activation of ABA biosynthesis genes (*ABA3, AAO3*) following drought stress or ABA treatment (Xiong et al. 2001b). A novel U-box E3 ubiquitin ligase SAUL1 (SENESCENCE-ASSOCIATED E3 UBIQUITIN LIGASE 1) also interacts with and targets AAO3 for ubiquitin-dependent degradation via the 26S proteosome (Raab et al. 2009). Analysis of wild-type plants and *saul1* mutants further revealed premature senescence in *saul1* mutants could be linked to elevated ABA biosynthesis corresponding to activation of AAO3 (Raab et al. 2009). From genetic and

biochemical studies SAUL emerged as a negative regulator of premature senescence (and ABA biosynthesis) by targeting AAO3 for degradation (Raab et al. 2009). From early work conducted with *sad1* and *saul1* mutants it is apparent ABA metabolism is likely subject to a suite of regulatory mechanisms which operate at the transcriptional, post-transciptional and even post-translational levels.

An emerging field of study in ABA metabolism surrounds the physiological relevance of ABA conjugates (ABA-GE) towards modification of intracellular and extracellular ABA levels (Sauter et al. 2002; Priest et al. 2006). Previously regarded as physiologically inactive forms of ABA which accumulate in tissues with age or stress treatments, increasing evidence suggests the compartmentalization and/or hydrolysis of ABA-GE is an important regulatory step in modifying intracellular and extracellular ABA levels. Biochemical and genetic studies conducted in barley (*Hordeum vulgare*) and *A. thaliana*, respectively, provide evidence that plants harbour β-glucosidases that specifically hydrolyze ABA-GE to physiologically active ABA (Dietz et al. 2000; Lee et al. 2006; Xu et al. 2012). In barley, Dietz et al. (2000) found extracellular washing fluids contained β-glucosidase activity capable of liberating ABA from ABA-GE conjugates at rates higher than 16-fold that detected in crude extracts of leaves. Intracellular β-glucosidases targeted to the endoplasmic reticulum (AtBG1) or vacuole (AtBG2) have also been identified in plants and are capable of hydrolyzing intracellular ABA-GE to ABA, which can subsequently be secreted into the apoplastic space of plant cells (Lee et al. 2006; Xu et al. 2012).

2.1.4 Abscisic acid signaling in plants

In the past 30-40 years, molecular genetic, biochemical, pharmacological and cell biology approaches have yielded a large number of loci (> 100) and secondary messenger molecules (e.g. $Ca^{2+,}$ cyclic nucleotides, reactive oxygen species, phospholipids) which participate in ABA signaling (Finkelstein et al. 2002). However, the most significant recent developments in the ABA field surround the identification of ABA receptor complexes and their subsequent relationship to type 2C protein phosphatases (PP2Cs) and SNF1-related protein kinases (subgroup 2) (SnRK2) which function as negative and positive regulators, respectively of ABA-induced gene expression (Cutler et al. 2010). Collectively, these discoveries have led to a core double-negative regulatory system which operates at the apex of the ABA signal transduction pathway (Cutler et al. 2010; Hubbard et al. 2010; Umezawa et al. 2010). Briefly, in the absence of ABA, PP2Cs are responsible for inactivating SnRK2s by direct dephosphorylation. However, in response to developmental or environmental cues, the presence of ABA initiates the interaction of ABA receptors (PYR/PYL/RCAR) with PP2Cs, resulting in PP2C inhibition and SnRK2 activation (Cutler et al. 2010; Umezawa et al. 2010).

Prior to advances in identifying ABA receptor complexes and their relationship to PP2Cs and SnRKs, the isolation and characterization of ABA response loci initiated with the premise that mutants disturbed in early steps of ABA biosynthesis or ABA signal transduction would be affected in all ABA-mediated processes (Koornneef et al. 1998). In *A. thaliana*, all ABA-insensitive mutants (*abi1–abi5, abi8*) were initially selected as seeds which germinated at ABA concentrations capable of inhibiting wild-type seeds (Koornneef et al. 1982; Giraudat et al. 1992; Finkelstein 1994; Koornneef et al. 1998).

Two of these ABA-insensitive loci (*ABI1* and *ABI2*) were found to encode serine/threonine protein phosphatases 2C enzymes. However, the three subsequent ABA insensitive loci, *ABI3*, *ABI4* and *ABI5*, each encode transcription factors representing the B3, AP2 and bZIP transcription factor families, respectively (Finkelstein et al. 2002). An important component of ABA's involvement in stress adaptation surrounds the observation that gene expression in response to environmental stresses (e.g. osmotic stress, cold, salinity) often correlates with the presence or synthesis of ABA (Chandler and Robertson 1994; Finkelstein et al. 2002).

Pathways contributing to upregulation of abiotic stress-responsive genes are frequently partitioned into ABA-dependent and ABA-independent pathways depending upon the extent to which the presence or absence of ABA is required to achieve full activation of promoters or target genes (Agrawal and Jha 2010). The initial functional characterization of *cis*-acting elements involved in ABA-responsive gene expression was achieved by isolating promoter elements of stress-inducible genes from wheat (*Triticum aestivum*), rice (*O. sativa*) and barley (*H. vulgare*) and subsequently assessing their modular role in transient expression systems (Leung and Giraudat 1998). From early studies a conserved ABA responsive element (ABRE) was identified that confers ABA-responsive gene expression when present in two copies and designated as an ABA response complex (ABRC). Utilizing ABREs as bait in a yeast one-hybrid system led to the identification of ABRE-binding proteins (ABF/AREB), a group of bZIP transcription factors (e.g. ABI5, ABF1, ABF2, ABF3, ABF4) that function as transcriptional activators of ABA response genes and require ABA for full activation (Finkelstein et al. 2002; Yoshida et al. 2010). Promoter analysis of differentially regulated *RD29A* and *RD29B*

genes in *A. thaliana* indicate that a single ABRE, in conjunction with a coupling element, may be sufficient for high levels of ABA-dependent gene expression (Narusaka et al. 2003; Nakashima et al. 2006).

Promoter analysis of stress-responsive genes also identified *cis*-elements and corresponding transcription factors involved in regulating ABA-independent gene expression programs associated with low temperature stress, heat shock stress, osmotic stress, drought and salinity (for review see Xiong and Zhu 2003). Two well-characterized *cis*-elements include the C-REPEAT/DEHYDRATION RESPONSE ELEMENT (CRT/DRE) bound by members of the EREB/AP2 family of transcription factors including CBF1/DREB1B, CBF2/DREB1C and DREB1A (Gilmour et al. 1998; Medina et al. 1999; Xiong et al. 2002b). The CBF1-3 transcription factors function as key upstream regulators of the cold response pathway in plants. However, additional members of the EREB/AP2 family including DREB2A, DREB2B and DREB2C fulfil analgous roles in directing gene expression programs associated with drought, osmotic, salinity and high temperature stresses (Liu et al. 1998; Nakashima et al. 2006; Lim et al. 2007).

Apart from characterized ABRE and CRT/DRE motifs identified in the promoter of ABA and stress-responsive genes, additional families of transcription factors (e.g. ABI3, MYC/MYB, NAC, WRKY) and their cognate DNA-binding motifs contribute towards the activation or repression of ABA and stress-responsive genes (Agrawal and Jha 2010). Recent studies continue to clarify the redundant, antagonistic and cooperative interactions between characterized and novel *cis* and *trans*-acting factors which function in ABA-dependent and ABA-independent signal transduction pathways (Yoshida et al.

2010). Other studies have revealed direct physical interaction amongst transcription factors representing ABA-dependent (ABF2, ABF3, ABF4) and ABA-independent pathways (DREB2C, DREB1A) (Lee et al. 2010). Kaplan et al. (2006) also revealed *cis*-elements assigned as ABRE activate transcription in response to Ca^{2+} oscillations. In a broader perspective it is proposed that the unique attributes of an individual stress signaling pathway (e.g. salinity, osmotic stress, low temperature) reside largely in the combination of sensors perceiving the given stimuli, the specificity encoded by secondary messengers (e.g. phospholipids, Ca^{2+} oscillations) and patterns of gene expression that are unique to a given stress (Xiong et al. 2002b).

2.1.5 Abscisic acid functions in the reproductive development of crop species

Given the importance of reproductive processes in ultimately determining the quantity and quality of yield attained by crop species, it is not surprising that a significant proportion of the work examining the role of ABA at this stage of the plant life cycle has been conducted upon economically important species such as corn (*Zea mays*), wheat (*T. aestivum*), rice (*O. sativa*) and soybean (*Glycine max*). Recent reviews also reinforce the critical role(s) which ABA exerts in directing the development and physiology of reproductive processes of monocot and dicot crops exposed to cold, heat and drought stress (Liu et al. 2005; Barnabàs et al. 2008; Thakur et al. 2010). Expression of male sterility within members of the *Brassicaceae* has also been linked to ABA, suggesting this hormone serves as a molecular determinant of functional genic (nuclear) male sterile lines utilized in rapeseed and canola breeding programs (Shukla and Sawhey 1994; Zhu et al. 2010).

Some of the earliest studies of ABA in crop plants revealed exogenous ABA could phenocopy the effects of drought stress in reducing pollen viability and seed set in wheat (Morgan 1980). Waters et al. (1984) also noted an interaction between ABA and sucrose levels in determining seed set in wheat. While elevated ABA levels tended to have a negative impact upon grain number per spikelet, the effects of ABA could be countered by applying sucrose. The authors considered that the effects of exogenous or endogenous ABA on reproductive failure of wheat may represent an indirect consequence of altered photosynthesis or transpiration which impair assimilate export from leaves or subsequent delivery of assimilates to reproductive structures (Waters et al. 1984). Profiling several hormones (ABA, IAA, cytokinins) in isolated reproductive structures (anthers, carpels) of wheat prior to fertilization revealed ABA levels were extremely dynamic (50 to 700 ng g^{-1} DW) within the tissue and developmental stage being sampled. Within individual spikelets, elevated levels of anther ABA tended to associate with floret positions failing to set seed (Lee et al. 1988). Comparable studies determined ABA contents were equally dynamic (2 to 400 ng g^{-1} DW) during later stages of reproductive development (e.g. grain filling) with ABA promoting seed maturation or directing assimilates and dry matter accumulation in wheat seeds (King 1976; Dewdney and McWha 1978).

Similar studies in soybean quantified ABA levels to ascertain the role of ABA in regulating the sugar status or growth rate of developing soybean seeds (Quebedeaux et al. 1976; Ackerson 1985). ABA levels in post-anthesis reproductive structures (pod wall, seeds) were highest in seeds (3000 ng g^{-1} FW) 20 days post-anthesis with peaks coinciding with the active period of seed growth and dry matter accumulation

(Quebedeaux et al. 1976). However, as seed development and maturation progressed to 60 days post-anthesis ABA levels declined to less than 10 ng g^{-1} FW. While pods contained lower levels of ABA (50 to 350 ng g^{-1} FW) relative to seeds a spike in pod ABA levels occurred 40-50 days post-anthesis and coincided with pod senescence (Quebedeaux et al. 1976). A subsequent study captured earlier stage reproductive development and analyzed ABA contents, carbohydrate status and invertase activity of flowers, pod walls and seeds (Ackerson 1985). Invertase content and activity were greatest for young actively growing reproductive structures at pollination whereas invertase activity sequentially declined in pods and seeds at later sample dates. Furthermore, in both flowers and pod walls, high invertase activity was associated with high levels of glucose relative to sucrose. The authors determined ABA could stimulate invertase activity in mature pods, but a clear relationship between ABA content and invertase activity did not exist during early stages of reproductive development (Ackerson 1985). From these results it was hypothesized that for specific reproductive structures endogenous ABA could be regulating acid invertase activity and hydrolysis of sucrose into hexoses such as glucose, which are preferential substrates for pod and seed growth (Ackerson 1985).

Shading soybean flowers was also employed to examine carbon and ABA partitioning between subtending leaves and reproductive structures of soybean (Yarrow et al. 1988). Isolated shading of reproductive organs 0 to 22 days after anthesis consistently reduced the ABA content of developing pods and also reduced photoassimilate partitioning from leaves. These observations led to the proposal that elevated ABA contents in reproductive organs, as a consequence of either endogenous synthesis or

translocation from leaves, may exert a role in preferential partitioning of photoassimilates to reproductive structures. However, the experimental approach employed did not enable a clear distinction of whether differences in assimilate partitioning could be attributed to events occurring at the sink (reproductive) or source (leaf) end of the transport pathway (Yarrow et al. 1988).

Beyond early studies examining the role of ABA in the reproductive development of wheat or soybean, interactions between ABA, ethylene and cytokinin underlie the physiological processes that direct whole-plant senescence, carbon mobilization, grain set and post-anthesis grain filling in monocot and dicot crops (Yang et al. 2002; 2003; Liu et al. 2004; Yang et al. 2006b; Travaglia et al. 2009; Liu et al. 2010). In rice and wheat, changes in ABA and cytokinin content of above or belowground structures were identified as correlative markers triggering senescence and remobilization of stored carbon (Yang et al. 2002; 2003). Agronomic treatments such as soil drying or altering nitrogen nutrition also accelerated or delayed whole-plant senescence, respectively, with elevated levels of ABA facilitating carbon mobilization and accelerating rates of grain filling (Yang et al. 2002; 2003; 2006). Comparable studies assessing pharmacological compounds applied to rice and wheat genotypes differing in grain-filling rates determined that ABA and ethylene acted in an antagonistic manner to regulate cell division and grain filling (Yang et al. 2006b). The ABA content, or ratio of ABA to aminocyclopropane-1-carboxylic acid (ACC), was also positively correlated with cell division and grain-filling rates in superior versus inferior spikelets (Yang et al. 2006b).

Recent molecular studies of reproductive failure in crop species (maize, rice, wheat, tomato) following environmental stress found transcriptional down-regulation of

key genes involved in carbohydrate metabolism and transport (e.g. invertases, monosaccharide transporters) serves as a key determinant of male or female gametophyte failure following cold, heat or drought stress (Dorion et al. 1996; Andersen et al. 2002; Koonjul et al. 2005; Oliver et al. 2005; 2007; Boyer and McLaughlin 2007; Jin et al. 2009). A subset of these studies identified ABA as a putative regulator of apoplasmic and symplasmic transport pathways mediating carbohydrate transport from photosynthetically active tissues to reproductive sinks such as flowers, fruits and seeds (Oliver et al. 2007; Jin et al. 2009; Ruan et al. 2010). Based upon genetic studies in *A. thaliana* interactions between ABA and sugar signaling pathways have been known for some time (Gibson 2004; Rolland et al. 2006) with several groups determining glucose-insensitive (*gin*) and sucrose-insensitive (*sin*) mutants are often allelic to ABA biosynthesis and signaling mutants (Arenas-Huertero et al. 2000; Laby et al. 2000; Huang et al. 2008).

2.1.6 Abscisic acid functions in the reproductive development of model species

Molecular studies of ABA metabolism and signaling in *A. thaliana* have largely focused on seed development or classical ABA responses such as seed germination, stomatal opening and closure or developmental arrest of young seedlings (Finkelstein et al. 2002; Nambara and Marion-Poll 2005). Early stages of reproductive development have received less attention although pre- and post-pollination reproductive structures are considered active sites of ABA metabolism and signaling (Peng et al. 2006; Priest et al. 2006). In localizing ABA during early stage of reproductive development, Peng et al. (2006) determined ABA preferentially accumulates in primordial or nursing cells (tapetum, integuments) responsible for delivering nutrients to actively differentiating

reproductive structures. The specific localization of ABA to nursing cells of male and female organs led to the hypothesis that in certain developmental contexts ABA acts in a positive fashion to direct assimilate flow or distribution (Peng et al. 2006).

Genetic studies also support a promotive role for ABA in reproductive development. For example, ABA-deficient mutants (*aba1, aba2*) display flowers and inflorescences of reduced stature in addition to smaller siliques with an increased frequency of unfertilized ovules or aborted embryos (Cheng et al. 2002; Barrero et al. 2005). Vegetative and reproductive phenotypes of ABA-deficient mutants can also be reverted through exogenous applications of ABA and suppression of ethylene biosynthesis (Sharp et al. 2000; Le Noble et al. 2004). Molecular analysis (RT-qPCR, promoter-GUS) of the *NCED* gene family in *A. thaliana* determined specific members (*NCED2, NCED5*, *NCED6*) display highly localized patterns of expression within immature floral buds, anthers and mature pollen grains (Tan et al. 2003). Hormone and transcriptional profiling studies further indicate reproductive organs (flowers, siliques) contain high levels of ABA, ABA catabolites (Priest et al. 2006) and express genes (*CYP707A1-A4*) encoding 8'hydroxylases at high levels (Saito et al. 2004).

As with ABA biosynthesis mutants, the majority of ABA response mutants in *A. thaliana* have been identified based upon ABA-resistant germination assays. However, relative to ABA biosynthesis mutants (*aba1, aba2),* most ABA response mutants display subtle phenotypes when grown under non-stressed conditions. However, an exception to this pattern occurs with *abi8* (*aba-insensitive 8*) mutants, initially identified based on ABA-resistant germination but subsequently displaying severe reproductive phenotypes

(delayed flowering and male sterility) and mis-regulation of sugar-mobilizing enzymes such as invertases and sucrose synthase (Brocard-Gifford et al. 2004).

The constitutive overexpression of ABRE Binding Factors 3 (ABF3) and 4 (ABF4) in *A. thaliana* resulted in hypersensitivity to ABA and sugars during seed germination and seedling development (Kang et al. 2002). Although *ABF3* and *ABF4* overexpressors were not examined in detail for reproductive phenotypes (short, stunted siliques), promoter-GUS analysis indicate native *ABF3* and *ABF4* expression is directed towards flowers, replum, funiculi and the silique abscission zone (Kang et al. 2002). Within male and female gametophytic tissues, closely related bZIP transcription factors (ABI5, ABF3) also target *AtSUC1*, a highly expressed H^+-dependent sucrose transporter (Feuerstein et al. 2010; Hoth et al. 2010). Promoter-truncation analysis revealed *AtSUC1* and *AtSUC2* contain *cis*-elements corresponding to characterized ABA-responsive elements (ABRE). Together, these observations suggest ABA-dependent bZIP transcriptions factors regulate aspects of sugar metabolism, transport or response in reproductive organs of *A. thaliana*.

The isolation of severe higher order mutants deficient in key positive (SnRK2.2, SnRK2.3, SnRK2.6) or negative regulators (HAB1, ABI1, ABI2, PP2CA) of the ABA signaling pathway indicate that under non-stressed conditions ABA signaling is critical for vegetative growth and reproductive development (Fujii and Zhu 2009; Fujita et al. 2009; Rubio et al. 2009). The phenotype of mutants with a constitutively activated ABA signaling pathway clearly indicate that hyperactivation of the ABA signaling pathway is detrimental towards plant growth and development under non-stressed conditions (Rubio et al. 2009). Collectively, the work with characterized ABA biosynthesis and signaling

mutants provide evidence that ABA metabolism and signaling are critical for vegetative and reproductive development.

2.2 Phospholipid metabolism and signaling

2.2.1 Phospholipid metabolism and signaling in eukaryotic organisms

In all eukaryotic organisms, phosphatidylinositol (PtdIns) metabolism generates a large and diverse group of myo-inositol based compounds which can broadly be partitioned into water soluble 'inositolpolyphosphates' and water insoluble membrane lipids or 'polyphosphoinositides' (Munnik and Vermeer 2010). Although polyphosphoinositides are membrane localized they posess polar head groups which protrude into the aqueous phase of cells and are accessed by lipid kinases and phosphatases to generate a diverse range of phosphoinositide (PI) species through addition or removal of phosphate groups located at various positions (D3, D4, D5) upon the inositol ring (Fig. 2.2) (Irvine and Schell 2001; Meijer and Munnik 2003). Phospholipid-based molecules are ubiquitous components of cellular membranes in all eukaryotic organisms. In comparison, inositol phospholipids represent approximately 10 to 15% of the phospholipid pool, with phosphoinositide (PI) species accounting for 0.5 to 1.5% of the total cellular phospholipid pool (Di Paolo and De Camilla 2006; Lemmon 2008; Thole and Nielsen 2008). However, this relatively small pool of lipid species exerts a disproportionately large influence upon signaling, membrane trafficking and organelle identity within eukaryotic cells (Irvine and Schell 2001).

In recent years the spatio-temporal and subcellular regulation of phosphoinositide metabolism within cells and preferential accumulation of phosphoinositide species to specific organelles (e.g. nucleus, plasma membrane, golgi apparatus) have been focused

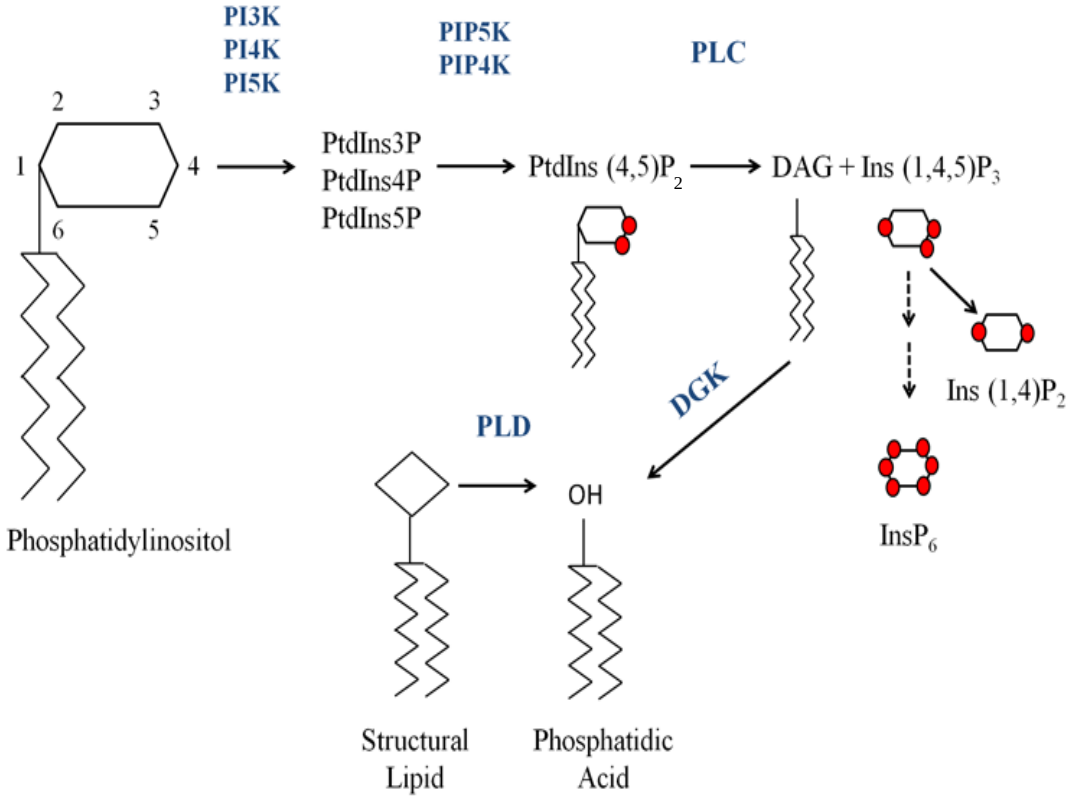

Fig. 2.2 Phosphoinositide metabolic and signaling pathway in plants. Lipid kinases and phosphatases generate distinct phosphoinositide species with phosphates (in red) located at various positions upon the inositol ring. Phospholipase C (PLC) enzymes hydrolyze specific phosphoinositide species to generate the secondary messenger molecules diacylglycerol (DAG) and inositol – 1,4,5 triphosphate ($InsP_3$). Phospholipase D (PLD) enzymes hydrolyze structural phospholipids to generate the lipid second messenger phosphatic acid (PA).

areas of research (Irvine 2003; Behnia and Munro 2005). While early studies of phospholipid signaling in eukaryotes were focused upon cellular events (e.g. ion channel activity, actin polymerization, membrane trafficking) occurring at the plasma membrane-cytosol interface, nuclear lipid metabolism and signaling are currently being examined as mediators of gene expression, chromatin remodeling and mRNA export (Irvine 2003; Monserrate and York 2010). Thus far, studies of nuclear lipid signaling have occurred predominantly in yeast and animal systems although increasing evidence points towards important roles for phospholipid metabolism and signaling in regulating nuclear processes in plant cells (Tan et al. 2007; Mishkind et al. 2009).

Phospholipid signaling has been implicated in root growth and root hair patterning, pollen development, proliferation and differentiation of vascular tissues, response to the hormones auxin and abscisic acid (ABA), in addition to contributing towards aspects of light and sugar signaling (Xue et al. 2009). Phospholipid-based signaling is also recruited to mediate the response of plant cells to abiotic stress or interactions with symbiotic or pathogenic microorganisms (Meijer and Munnik 2003).

Models of phospholipid-based signal transduction are heavily rooted in mammalian studies of phosphoinositide metabolism and signaling in addition to the isolation and characterization of proteins harbouring lipid-binding domains (Irvine and Schell 2001; Di Paolo and De Camilla 2006; Lemmon 2008). While generally accepted that phosphoinositide metabolism and signaling exert equally important roles in the function of plant cells, there is considerable sentiment that the 'language' of lipid signaling in higher plants differs significantly from that of other eukaryotic organisms (Worrall et al. 2003; Munnik and Vermeer 2010). In following sections distinguishing

features of phosphatidylinositol metabolism and signaling are presented with specific emphasis on changes in phospholipid metabolism following environmental stress or in response to the hormone abscisic acid (ABA). The function of well characterized (e.g. C2, PH) and putative (GRAM) lipid binding domains are also discussed.

2.2.2 Phosphatidylinositol metabolism and signaling in plants

In eukaryotes, *myo-inositol* phosphatidylinositol synthase is a key rate-limiting enzyme responsible for initial conversion of glucose-6-phosphate to *myo*-inositol-3-phosphate (Ins3P). In turn Ins3P is dephosphorylated by inositol monophosphatase to form free inositol, which is subsequently transferred to a glycerol backbone by phosphatidylinositol synthase to generate phosphatidylinositol, the base molecule of all inositol-based phospholipids (Fig. 2.2). Once phosphatidylinositol has been formed, a specific group of phosphoinositide kinases (PI3K, PI4K, PI5K) can phosphorylate the inositol ring at various position (D3, D4, D5) to form polyphosphoinositide species such as PtdIns3P, PtdIns4P or PtdIns5P. In sequential fashion these newly formed polyphosphoinositides can be further phosphorylated through another subset of phosphoinositide kinases (PIP5K, PIP4K) to generate species such as PtdIns (4,5)P_2. At this juncture phosphorylation of the inositol head group of various PtdIns species results in the generation of one of seven polyphosphoinositides. Animals, yeast and plants also possess a suite of phosphatases (3PTase, 4PTase, 5PTase) capable of removing phosphates from the D3, D4 and D5 position of the inositol ring (Majerus et al. 1999; Munnik and Testerink 2009). Utilizing the available suite of kinases and phosphatases eukaryotic cells manipulate phosphatidylinositol metabolism to either form or degrade

specific signaling molecules in response to internal or external stimuli (Meijer and Munnik 2003).

In addition to lipid kinases and phosphatases, eukaryotic phospholipid signaling also recruits various phospholipase isoforms (e.g. phospholipase C, phospholipase D, phospholipase A) to hydrolyze phospholipid substrates into secondary messenger molecules (Munnik and Testerink 2009). In mammalian systems, a classic lipid-signaling pathway involves the recruitment of phospholipase C (PLC) to hydrolyze PtdIns $(4,5)P_2$ into the secondary messenger molecules inositol-1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) which trigger the release of Ca^{2+} from intracellular stores or activate members of the protein kinase C family (PKC), respectively (Berridge 1993). The search to identify a ubiquitous PLC-based signaling pathway in eukaryotic organisms also led plant scientists to propose that PLC and IP_3 exert similar roles as secondary messengers relative to ABA signaling in plant cells (Sanchez and Chua 2001; Mills et al. 2004). However, enzymes and secondary messengers involved in plant lipid signaling are likely unique from those in other eukaryotes (van Leeuwen et al. 2004). For example, in plants inositol hexakisphosphate (IP_6), rather than IP_3, has been implicated as a more potent secondary messenger capable of triggering Ca^{2+} release from intracellular stores during ABA-mediated stomatal closure (Lemtiri-Chlieh et al. 2003). Furthermore, plants do not contain homologs to mammalian IP_3 receptors or the PKC family (Krinke et al. 2007). Rather, once DAG is formed by plant PLCs, it is rapidly phosphorylated by a family of diacylglycerol kinase (DGK) enzymes to generate phosphatidic acid. Therefore, as opposed to utilizing IP_3 and DAG as secondary messengers as observed for mammalian PLC-based signaling, plants, fungi and slime molds may have evolved a

form of PLC signaling which produces IP_6 and PA as key secondary messengers (Meijer and Munnik 2003). In addition to PLC-based formation of PA in plants, phospholipase D (PLD) has emerged as a critical enzyme involved in phospholipid signaling. PLD isoforms are responsible for mediating the hydrolysis of structural phospholipids (e.g. phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol) to phosphatidic acid, providing an alternative route for phosphatidic acid synthesis. In comparison to mammalian or yeast genomes which contain one or two PLD genes, the genomes of higher plants contain a more diverse PLD family, with twelve members identified in the sequenced genome of *A. thaliana* (Meijer and Munnik 2003). In comparison, for PLC genes, mammalian genomes contain members from each of six PI-PLC subfamilies (β, γ, δ, ε, η and ξ) whereas all plant PI-PLCs belong to the PLCξ subfamily which lack N-terminal pleckstrin homology (PH) domains and display incomplete EF motifs involved in Ca^{2+} binding (Munnik and Testerink 2009). In spite of these observations, plant PI-PLC isoforms continue to exhibit Ca^{2+} dependent hydrolysis of phosphoinositide species with nine functional PI-PLC equivalents identified in the sequenced genome of *A. thaliana* (Hunt et al. 2004; Tasma et al. 2008).

In plants, PLD-generated phosphatidic acid may be phosphorylated to diacylglycerolphosphate in a reaction mediated by phosphatidic acid kinase. Although genes encoding phosphatidic acid kinase have yet to be identified, this represents a plausible mechanism through which PA can be terminated. In spite of differences which have emerged between phospholipid metabolism and signaling in mammals and higher plants, many components remain conserved across all eukaryotes species. For example, recent studies of an external calcium receptor (CAS) in plants (Tang et al. 2007; Nomura

et al. 2008) suggest IP_3 is still capable of mediating intracellular Ca^{2+} release in plant cells. More importantly, while some controversy currently surrounds the exact subcellular location (plasma membrane or chloroplast) of plant CAS proteins, chloroplasts exert a prominent role in Ca^{2+} storage and signaling in plant cells whereas mitochondria and the endoplasmic reticulum (ER) fulfill analogous roles in animal cells (Webb 2008). As activities and storage of Ca^{2+} are often coupled to phospholipid metabolism and signaling in eukaryotic cells, it is plausible Ca^{+2} and phospholipid signaling pathways of plants have diverged from other eukaryotic organisms as a means of supporting the unique physiology and structure of autotrophic plant cells (Webb 2008).

2.2.3 GRAM domain genes in eukaryotic organisms

GRAM (**G**lucosyltransferase, **Ra**b-like GTPase activators and **M**yotubularins) domains were initially identified by Doerks et al. (2000) through computational analysis of animal, plant and fungal proteins. However, upon crystallographic analysis of human MTMR2, a dual specific protein tyrosine phosphatase, it became apparent the GRAM domain was part of a larger motif present within a pleckstrin homology (PH) domain fold (Begley and Dixon 2005). PH domains are well-characterized PI-binding domains and existing biochemical studies conducted in eukaryotic systems indicate this is a likely function of the GRAM domain (Tsujita et al. 2004; Slavsgold et al. 2005; Lorenzo et al. 2006; Yamashita et al. 2006). Other studies have proposed dimerization modules for GRAM domain proteins with one GRAM domain required to bind phosphoinositides, but dimerization of two GRAM domain proteins required for membrane targeting or interaction with partner proteins in the membrane (Berger et al. 2003).

Detailed molecular and biochemical studies of eukaryotic GRAM domain proteins have focused on the myotubularin (MTM) family in humans (*Homo sapiens*) or autophagy-related proteins in yeast (Begley and Dixon 2005; Yamashita et al. 2007; Vergne and Deretic 2010). The myotubularin family (MTM1 and MTMR1-MTMR13) of phosphatases are large multi-domain proteins which preferentially utilize phosphoinositide (PI) species, as opposed to phosphoproteins, as physiological substrates. Very recently, catalytically active MTM homologs were identified in higher plants. However, relative to the 15 member family of MTM proteins in humans, *A. thaliana* contains only two MTM proteins (Vergne and Deretic 2010; Ding et al. 2012). In humans, mutations in two genes (*MTM1* and *MTMR2*) have been associated with X-linked myotubular myopathy or Charcot-Marie-Tooth disease type 4B (Begley and Dixon 2005). Functional studies in yeast and humans further revealed GRAM proteins mediate the synthesis (kinase) and degradation (phosphatase) of phosphoinositide species (PI3P, PI4P) mediating the induction of autophagy (Oku et al. 2003; Yamashita et al. 2006; 2007; Vergne et al. 2009; Zou et al. 2012). Autophagy, meaning "self-eating", is a universal feature of eukaryotic cells through which organelles, damaged proteins or cytosolic macromolecules are degraded to recycle nutrients. In all eukaryotic organisms including plants, autophagy is characterized by the formation of distinct organelles (e.g. autophagosomes) within the cell (Bassham 2007; Yang and Klionsky 2010).

The first GRAM domain sequence (*ABA45*) identified in plants was initially isolated from a cDNA library constructed from barley aleurone tissues treated with ABA and subsequently screened with anti-idiotypic (AB2) antibodies to identify putative ABA-binding proteins (Liu et al. 1999). Northern blot analysis revealed ABA treatments

increased mRNA levels of *ABA45*, with transcripts accumulating to high levels in reproductive tissues (Liu et al. 1999). In *A. thaliana* Finkelstein and Rock (2002) originally identified an eight member family of GRAM genes with significant identity to barley *aba45*, although more recent estimates suggest 13 and 17 GRAM genes exist in the sequenced genomes of *A. thaliana* and *O. sativa*, respectively (Jiang et al. 2008). RT-qPCR analysis (*Oryza sativa*) and publicly available microarray data (*Arabidopsis thaliana*) further indicate members of the GRAM gene family are differentially expressed (up- or down-regulated) in response to ABA, nutrient deprivation or environmental stress (Jiang et al. 2008).

In *A. thaliana* and *O. sativa*, most members of the GRAM domain gene family remain uncharacterized. In *A. thaliana*, ***G****LABRA2* ***E****XPRESSION* ***M****ODULATOR* (*GEM*) is one characterized member of the GRAM family which functions to couple cell cycle and cell fate decisions during epidermal root hair patterning (Caro et al. 2007). Based upon yeast-two hybrid screens GEM was initially isolated as a putative interactor with CDT1, a characterized DNA replication protein in *A. thaliana* (Castellano et al. 2004; Caro et al. 2007). Further analysis revealed GEM not only interacts with CDT1, but also forms protein-protein interactions with components of a multimeric transcription factor complex (TTG1-GL3-EGL3-WER) involved in root epidermal patterning. Chromatin immunoprecipitation experiments also revealed GEM was recruited to the promoter region of key epidermal patterning genes *GLABRA2* and *CAPRICE*. Through analysis of loss-of-function (*gem1*) and gain-of-function (GEM^{OE}) backgrounds it was revealed that GEM was capable of modifying the epigenetic status of *GLABRA2* and *CAPRICE* promoters. Through direct physical interaction with cell cycle (CDT1) and patterning

(TRANSPARENT TESTA GLABRA1) proteins GEM was proposed to function as a novel integrator of cell division and cell fate decisions in plants (Caro et al. 2007).

A second member of the GRAM domain family subject to functional studies is VASCULAR ASSOCIATED DEATH 1 (VAD1) (Lorrain et al. 2004; Bouchez et al. 2007). The *vad1-1* mutant displays a light-conditional appearance of hypersensitive-response lesions along the vascular system (Lorrain et al. 2004) and subsequent genetic studies revealed ethylene biosynthesis and signaling are required for cell death and defense phenotypes in *vad1-1* mutants in addition to promoting *VAD1* expression during the hypersensitive response (Bouchez et al. 2007). More recently, utilizing a high throughput proteomic screen, Choi and Hwang (2011) isolated a pepper (*Capsicum annuum*) GRAM domain gene, *ABA-RESPONSIVE1* (*CaABR1*), which is expressed in reponse to ABA treatment and pathogen attack. Functional studies determined *CaABR1* participates in cell death and defense signaling, reactive oxygen species production and ABA-salicylic acid antagonism. The GRAM domain of ABR1 was also essential for cell death responses and nuclear localization. In *A. thaliana*, ROSCOVITINE RESISTANT 1 (ROR1) is an additional member of the GRAM gene family which has been functionally characterized. Roscovitine resistant mutants (*ror-1* to *ror-5*) were initially isolated from an activation tagged population utilized in a genetic screen aimed at identifying novel components of the cytokinin glucosylation pathway in plants (Dwivedi et al. 2010). Comparative hormone profiling of wildtype and *ror-1* mutants revealed bioactive cytokinins were not significantly different between lines. However, in *ror-1* mutants, cytokinin precursors were significantly depleted whereas levels of cytokinin *N*-glucosides (inactive cytokinins) were enhanced (Dwivedi et al. 2010). RT-qPCR studies further

revealed *ROR1* was cytokinin-inducible with the phenotype of *ror-1* mutants (activation tagged) associated with a corresponding hyperactivation of *ROR1* transcripts (Dwivedi et al. 2010). Collectively the functional analysis of *ror-1* mutants indicated *ROR1* was a novel cytokinin-inducible gene which activates a putative *N*-glucosylation pathway in plants.

Overall, the combined cell biology, genetic and biochemical approaches employed in characterizing eukaryotic GRAM genes have provided critical insight into approaching the functional studies of analogous genes in plants. Across both monocot and dicot species, genomic and proteomic studies provide ample evidence GRAM genes are responsive to ABA and various forms of environmental stress (Liu et al. 1999; Hoth et al. 2002; Yazaki et al. 2004; Riccardi et al. 2004; Lee et al. 2005; Jiang et al. 2008). However, relative to GRAM genes of *H. sapiens*, *M. musculus*, *D. melanogaster* and *S. cerevisiae*, plant counterparts do not display the same abundance of non-catalytic and catalytic domains (e.g. FYVE, TBC) (Doerks et al. 2000; Jiang et al. 2008). Nonetheless, several members of the GRAM gene family in *A. thaliana* and *O. sativa* have retained the C2 ($Ca^{2+/}$phospholipid) domain which functions to couple Ca^{2+} and phosphoinositide signaling pathways in plants (Zheng et al. 2000; Cho 2001).

2.3 Objectives

In the following thesis our main objective surrounded the isolation and characterization of a novel GRAM domain gene, ***GE****M-****R****ELATED* ***5*** (*GER5*), hypothesized to function in abscisic acid (ABA) metabolism and signaling during plant growth, development and stress responses. In testing this hypothesis, a reverse genetic

approach was employed to generate mutant or transgenic lines with altered levels of *GER5* expression. Based upon reproductive phenotypes which emerged in mutant and transgenic lines, a second study was initiated to assess the transcriptional regulation of GRAM genes in response to abiotic stress or within mutant backgrounds deficient in ABA metabolism or signaling. As several eukaryotic GRAM genes function in autophagy, and ABA has emerged as a plant-specific regulator of autophagy, we hypothesized core *A*u*T*opha*G*y-related (*ATG*) and GRAM domain genes were similarly regulated by ABA-dependent and ABA-independent signaling pathways. To date, the majority of molecular studies examining ABA metabolism and signaling have focused on mature seeds or vegetative tissues. Based upon reproductive phenotypes which emerged from reverse genetic studies of *GER5*, we hypothesized rapidly growing reproductive organs would be characterized by novel aspects of ABA metabolism and homeostasis. To evaluate this hypothesis, a third study was conducted to assess the transcriptional response of key genes involved in ABA biosynthesis, catabolism, transport and how these genes were differentially regulated within cauline leaves, inflorescence meristems and developing siliques exposed to cold and heat stress.

3.0 GEM-RELATED 5 (GER5), an ABA and stress-responsive GRAM domain protein regulating seed development and inflorescence architecture

[Baron KN, Schroeder DF, Stasolla C, submitted to Planta, January 2013]

3.1 Abstract

We have identified an abscisic acid (ABA) and stress-responsive GRAM (**G**lucosyltransferases, **Ra**b-like GTPase activators and **M**yotubularins) domain protein GER5 (**GE**M-**R**ELATED **5**) closely related to GEM (**G**LABRA2 **E**XPRESSION **M**ODULATOR), a novel regulator of cell division and cell fate determination in epidermal cells. A loss-of-function T-DNA line (*ger5-2*) and transgenic lines silencing ($GER5^{RNAi}$) or overexpressing ($GER5^{OE}$) *GER5* displayed several defects in reproductive development affecting seed and embryo development. RNA *in situ* studies revealed *GER5* and related GRAM genes (*GEM* and ***GEM-RELATED 1*** (*GER1*)) have both overlapping and unique expression domains in male and female reproductive organs. Hormone immunolocalization experiments further indicated *GER5* transcripts preferentially localize to reproductive tissues which accumulate ABA. Expression analysis revealed members of the GRAM family (*GER5, GER1, GEM*) display tissue-specific expression patterns and are responsive to phytohormones and abiotic stress, as well as genetic lesions (*aba1, aba2, ctr1*) affecting ABA biosynthesis or ethylene signaling. Mature seeds of *ger5-2* mutants also exhibited reduced sensitivity to ABA during seed germination assays. Microarray analysis of aborting and developing seeds isolated from *ger5-2* mutants revealed underlying transcriptional changes in carbohydrate metabolism, hormone signaling and catabolic processes (e.g. protein degradation,

autophagy). Taken together, our results indicate ABA-responsive GRAM genes play a novel role in regulating the reproductive development of plants, and raise intriguing questions regarding the functional relationship between members of the GRAM gene family.

3.2 Introduction

The plant hormone abscisic acid (ABA) mediates an array of agronomically important processes including seed maturation and inductive dormancy, stomatal opening and closure, and the transcriptional response of plants to cold, heat and drought stress (Finkelstein et al. 2002). Recent efforts in the ABA field have focused on a core ABA signaling complex through which ABA binds to receptors (PYR/PYL/RCAR) and disrupts the physical interaction between phosphatase 2Cs (PP2Cs) and SNF1-related protein kinases 2 (SnRK2s), which function as negative and positive regulators of the ABA signaling pathway, respectively (Cutler et al. 2010; Klingler et al. 2010). Inhibition of PP2Cs by ABA receptors enables SnRK2s to be phosphorylated, subsequently leading to activation of the ABF/AREB family of transcription factors which drive the expression of ABA-responsive genes (Klingler et al. 2010). In spite of considerable progress made in clarifying upstream components of the ABA signal transduction pathway, there are molecular, cellular and physiological roles for ABA in plant growth, development and stress response which have yet to be fully elucidated (De Smet et al. 2006; Yang et al. 2006a; Ton et al. 2009).

In plants, relatively little is known regarding the function of GRAM (**G**lucosyltransferases, **Ra**b-like GTPase activators and **M**yotubularins) domain genes, although several members appear responsive to ABA or various forms of abiotic or biotic

stress (Liu et al. 1999; Hoth et al. 2002; Riccardi et al. 2004; Yazaki et al. 2004; Lee et al. 2005; Jiang et al. 2008; Choi and Hwang 2011). However, the precise function of GRAM domain genes in ABA-mediated responses or signaling remains obscure.

In *Arabidopsis thaliana*, *VASCULAR ASSOCIATED DEATH1 (VAD1)* encodes a GRAM domain protein implicated in cell death and defense responses associated with vascular tissues. *VAD1* expression occurs at low levels in healthy plants but increases rapidly in response to pathogen infection (Lorrain et al. 2004). Genetic analysis revealed salicylic acid and ethylene were key determinants regulating the propagation of cell death in *vad1* mutants (Lorrain et al. 2004; Bouchez et al. 2007).

In a high-throughput proteomic screen, Choi and Hwang (2011) identified a pepper (*Capsicum annuum*) GRAM domain protein, ABSCISIC ACID-RESPONSIVE 1 (CaABR1) that was highly responsive to pathogen infection and ABA treatment. Subsequent functional analyses revealed the GRAM domain of ABR1 was necessary for nuclear localization and initiation of cell death responses following pathogen infection. A working model was proposed through which ABR1 modulates antagonistic interactions between ABA and salicylic acid to influence reactive oxygen species (ROS) production and cell death during host-pathogen interactions (Choi and Hwang 2011).

In yeast two-hybrid screens, a novel GRAM domain protein, **G**LABRA2 **E**XPRESSION **M**ODULATOR (**GEM**), was found to interact with CDT1, a conserved nuclear protein involved in DNA replication (Caro et al. 2007). Protein-protein interaction and chromatin immunoprecipitation studies further revealed GEM physically interacts with **T**RANSPARENT **T**ESTA **G**LABRA **1** (TTG1) and could also modify the epigenetic status of the epidermal patterning genes *CAPRICE* and *GLABRA2* (Caro et al.

2007). Taken together, GEM was proposed to function as a novel regulator of cell division and cell fate determination in plants.

Based on identity with barley (*Hordeum vulgare*) *ABA45*, the first ABA-responsive GRAM domain sequence identified in plants (Liu et al. 1999), and CaABR1, an ABA-responsive GRAM domain protein characterized in pepper (Choi and Hwang 2011), we sought to functionally characterize the role of homologous *A. thaliana* genes in plant growth, development and stress responses. Here we demonstrate that a mutant (*ger5-2*) and transgenic lines ($GER5^{RNAi}$, $GER5^{OE}$) with altered levels of *GER5* expression display defects in seed development, silique morphology and inflorescence architecture. RNA *in situ* hybridization studies indicate *GER5* and related GRAM genes (*GER1, GEM*) function during early stages of male and female gametophyte development. We also demonstrate *ger5-2* mutants have altered ABA responses during seed germination assays. Microarray analyses of aborting seeds isolated from *ger5-2* mutants revealed corresponding changes in carbohydrate metabolism, hormone signaling and catabolic processes. Taken together, our results indicate *GER5* and closely related GRAM domain genes (*GER1, GEM*) play an important role in the reproductive development of plants.

3.3 Materials and methods

3.3.1 Cloning, generation of transgenic plants, and genetic stocks

Mutant seed stocks used in the current study were obtained from the Arabidopsis Biological Resource Centre (ABRC), Nottingham Arabidopsis Stock Centre (NASC), or kindly provided by Selina Costas and Crisanto Gutierrez (Alonso et al. 2003; Rosso et al. 2003; Caro et al. 2007). A homozygous T-DNA insertion line (GABI_783H10) obtained

from NASC was named *ger5-2*. Oligonucleotide primers utilized for genotyping, cloning and gene expression analysis are described in Table 3-1S. Hormone biosynthesis and signaling mutants (Col-0) used in the current study (*aba1-5*, *aba2-1*, and *ctr1-1*) were obtained from the ABRC.

For generation of transgenic lines the full-length coding region of *GER5* (GenBank accession: NM_121232.3) was isolated from a full-length cDNA library (CD4-34 from ABRC), cloned into the pDONR entry vector (Invitrogen), and then sequenced. Using the Gateway recombination system (LR clonase) *GER5* entry clones were recombined with binary destination expression vectors (pK2GW7 and pK7GWIWG2(I)) to generate hairpin (RNAi) and overexpression (OE) constructs (Karimi et al. 2002). Constructs were then introduced into *Agrobacterium tumefaciens* (strain GV3101) via electroporation. Arabidopsis plants (Col-0) were subsequently transformed using the floral dip method (Clough and Bent 1998). For each construct 10-15 independently transformed seedlings were identified on kanamycin containing medium, transferred to soil, grown to maturity and allowed to self-pollinate. Progeny of self-pollinated transformants (T1) giving an approximate 3:1 (resistant:susceptible) segregation of the kanamycin marker were allowed to self until homozygous T3 plants were obtained. Cycling conditions, reagents and oligonucleotides utilized for cloning, RT-PCR and RT-qPCR analyses of mutant and transgenic lines and gene expression studies are provided in Table 3.1S and Appendix 8.1.

3.3.2 General histology and Hoyer's clearing

For general histology and structural analysis, developing seeds were processed, sectioned and stained with periodic-acid Schiff (PAS) reagent (Sigma) for total

carbohydrates, and counterstained with toluidine blue (TBO) for general histology according to methods described in Baron et al. (2012). For Hoyer's clearing, isolated seeds were cleared as described in Liu and Meinke (1998) and examined using an AxioImager fluorescence microscope (Zeiss) equipped with Nomarksi optics.

3.3.3 Growth conditions, phytohormone and stress treatments

Arabidopsis thaliana (Col-0) seeds were surface sterilized (70% EtOH, 0.5% Triton) and placed on sterile plates. Plates were stratified for 2d at 4℃ in darkness, placed under a light bar at a constant temperature of 22 ± 1.5℃, and provided 140 ± 20 μmol $m^{-2}s^{-1}$ from cool white fluorescent lamps. Following germination seedlings at the 4 leaf stage were transplanted in 8.5 cm^2 square pots containing media (LA4 mix, aggregate plus, SunGro Sunshine, Seba Beach, AB). After transplanting, flats of seedlings were transferred to growth chambers (Model GC-20, Enconair, Winnipeg, MB) providing constant 22℃ ± 1.0℃ on a 16 h (day):8 h (night) cycle and 70% relative humidity.

For phytohormone treatments, rosette leaves and entire inflorescences (stage 6.3-6.5, Boyes et al. 2001) were sprayed twice (24 and 2 h) before destructive harvests with solutions (50 mL) containing (±) cis, trans-ABA (30 μM) or GA_3 (10 μM) in addition to 0.075% Triton X-100. Control plants were similarly treated with 0.075% Triton X-100 before tissues (entire inflorescence) were isolated for RT-qPCR analysis. For abiotic stress experiments temperature treatments were applied as described in Baron et al. (2012). Briefly, wild-type plants (Col-0) grown at 22℃ were ramped at 2°C/h to target temperatures (0°C=cold, 37℃=heat) and exposed to 24 h of stress prior to tissue sampling. For temperature stress experiments and analysis of hormone

biosynthesis/signaling mutants grown at 22°C under ideal conditions, reproductive tissues were harvested and analyzed separately by RT-qPCR according to Baron et al. (2012). For analysis of GRAM gene expression during seedling development, tissues were isolated from seedlings grown on agar plates. For mature seeds, RNA was extracted using a modified TRIzol-based protocol (Meng and Feldman 2010). In the current study, RT-qPCR data was analyzed according to the comparative C_t ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak 2008) using *PP2A* (At1g13320) as reference gene (Czechowski et al. 2005).

3.3.4 Seed germination assays

For seed germination assays, age-matched seeds (grown under 22°C conditions described above, harvested and stored in parallel) of wild-type (Col-0), *ger5-2* mutant and *aba2-1* lines were surface-sterilized and sown on half-strength Murashige and Skoog (MS) medium containing 1% sucrose, 0.8% agar and concentrations of ABA ranging from 0 to 5.0 μM. Separate seed germination assays were conducted using compounds (paclobutrazol, fluridone, uniconazole) known to modify metabolism or signaling of ABA and GA. In all cases, seeds were stratified at 4°C for 2 days prior to assessment under light bar growth conditions described above.

3.3.5 RNA *in situ* hybridization and ABA immunolocalization

RNA *in situ* hybridization experiments were conducted according to the DIG Application Manual for In Situ Hybridization (Roche Diagnostics, Manheim, Germany) with the following modifications. Reproductive tissue samples were fixed in 4% paraformaldehyde dissolved in 1x PBS and fixed overnight at 4°C. Samples were then dehydrated in an ethanol series (4°C) followed by changes in xylene (room temperature) and paraffin wax (55°C) (Paraplast, Fisherbrand). After orienting samples, paraffin

blocks were allowed to harden, stored at 4°C, and sectioned (8 μm) using a rotary microtome. For generation of DIG-labelled probes *GER5* (At5g13200*), GER1* (At1g28200, GenBank accession: NM_102585) and *GEM* (At2g22475, GenBank accession: NM_127813.2) were isolated from cDNA derived from inflorescence meristems, cloned into the pGEM T-Easy vector (Promega), and then sequenced. M13 forward and M13 reverse primers were utilized to generate PCR products containing full-length versions of *GER5*, *GER1* and *GEM* which incorporated T7 and SP6 primer sites. Sense and antisense DIG-labelled probes for *GER5*, *GER1* and *GEM* were transcribed using T7 and SP6 polymerases (Roche Diagnostics). DIG-labelled probes were subsequently quantified on a spectrophotometer (ND-1000, Thermo Scientific) and incorporation of label verified by dot blot analysis.

ABA localization studies were conducted according to Peng et al. (2006) and Gong et al. (2006) with slight modifications. For detailed description of procedures utilized for ABA immunolocalization see Appendix 8.1. In the current study, a key step in obtaining signal specificity and reducing background levels surrounded pre-treatment of secondary antibodies with reproductive tissues of *A. thaliana* as per Gong et al. (2006). Controls were obtained by omitting primary monoclonal antibody or pre-treated secondary antibody.

3.3.6 RNA extraction, microarray hybridization and scanning

For each of three seed categories (wild-type = WT, homozygous *ger5-2* mutant green = HG, homozygous *ger5-2* mutant white = HW), 50-60 seeds (6 tubes per genotype/phenotype) were excised from staged siliques (10-14 DAP) and immediately submerged in liquid N_2. Total RNA (500-2000 ng total) was extracted from pooled seeds

using an Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA, USA) and assessed for integrity on a spectrophotometer. RNA samples (4 per seed category) were then amplified and reverse labeled with Cy3 and Cy5 dyes using an RNA ampULSe Kit (Kreatech Biotechnology, Amsterdam, The Netherlands). During amplification and labeling, amplified RNA was assessed for integrity and degree of labeling using both gel electrophoresis and spectrophotometry (ND-1000, Thermo Scientific). Arabidopsis oligonucleotide arrays (AROS Version 3.0) were utilized for microarray hybridizations (http://ag.arizona.edu/microarray/methods.html). For detailed description of microarray procedures see Appendix 8.1.

3.3.7 Statistical analysis and microarray validation

In the current study, a mixed model approach developed by Wolfinger et al. (2001) was utilized to assess differences in gene expression amongst seed categories from *ger5-2* mutant (HG = homozygous *ger5-2* green, HW = homozygous *ger5-2* white = white) or wild-type (WT = wild-type green) plants. The experimental design corresponded to a three-way direct comparison of seed categories with 12 Cy3-labelled aRNAs and 12 Cy5-labelled aRNAs hybridized in a dye-swap arrangement against 12 microarray slides (Yang and Speed 2002). For detailed description of procedures and workflow used see Appendix 8.1.

3.4 Results

3.4.1 *GER5* expression influences seed development and inflorescence architecture

The current study was undertaken to evaluate the function of *GER5* (***GE****M-****R****ELATED* ***5***), an uncharacterized member of a putative ABA-responsive gene family

(Fig. 3.1S) and Arabidopsis homolog of barley *aba45* and pepper *CaABR1* (Liu et al. 1999; Jiang et al. 2008; Choi and Hwang 2011). To study the function of *GER5* in plant development, we identified a single insertion mutant (GABI_783H10) from the GABI-KAT collection. From segregating T3 lines, we identified plants homozygous for the T-DNA insertion by PCR genotyping and named this mutant *ger5-2*. In addition, we generated transgenic Arabidopsis lines that silence or overexpress *GER5* by introducing constructs under the control of the 35S promoter. The intron-exon structure of *GER5* (At5g13200) and positioning of oligonucleotide primers utilized to characterize mutant and transgenic lines are shown in Fig. 3.1a, as well as the T-DNA insertion site associated with the *ger5-2* mutant allele. Characterization of *ger5-2* mutants by PCR and RT-qPCR revealed a complete loss of full length transcripts spanning the T-DNA insertion site (Fig. 3.1b,c). However, an exon 1-specific primer set revealed partial *GER5*-derived transcripts upstream of the T-DNA insertion site in *ger5-2* mutants (Fig. 3.1b), suggesting *ger5-2* is a partial loss-of-function allele. Using RT-qPCR to characterize gene expression in wild-type plants, overexpression lines ($GER5^{OE}$) and *ger5-2* mutants revealed abundant levels of transgene-derived transcript in $GER5^{OE}$ lines (Fig. 3.1c). For lines carrying the *GER5*-derived hairpin construct, ($GER5^{RNAi}$), RT-qPCR experiments revealed reduction of *GER5* endogenous transcript (Figure 3.1d). Therefore, $GER5^{RNAi}$ lines were characterized as loss-of-function knockdown alleles.

Mutant and transgenic lines evaluated in the current study displayed numerous phenotypes affecting the reproductive stage of plant development (Fig. 3.2, Fig. 3.1S). Across lines examined defects in seed development and seed abortion emerged as the most prominent phenotype associated with loss-or-gain of *GER5* function (Fig. 3.2a).

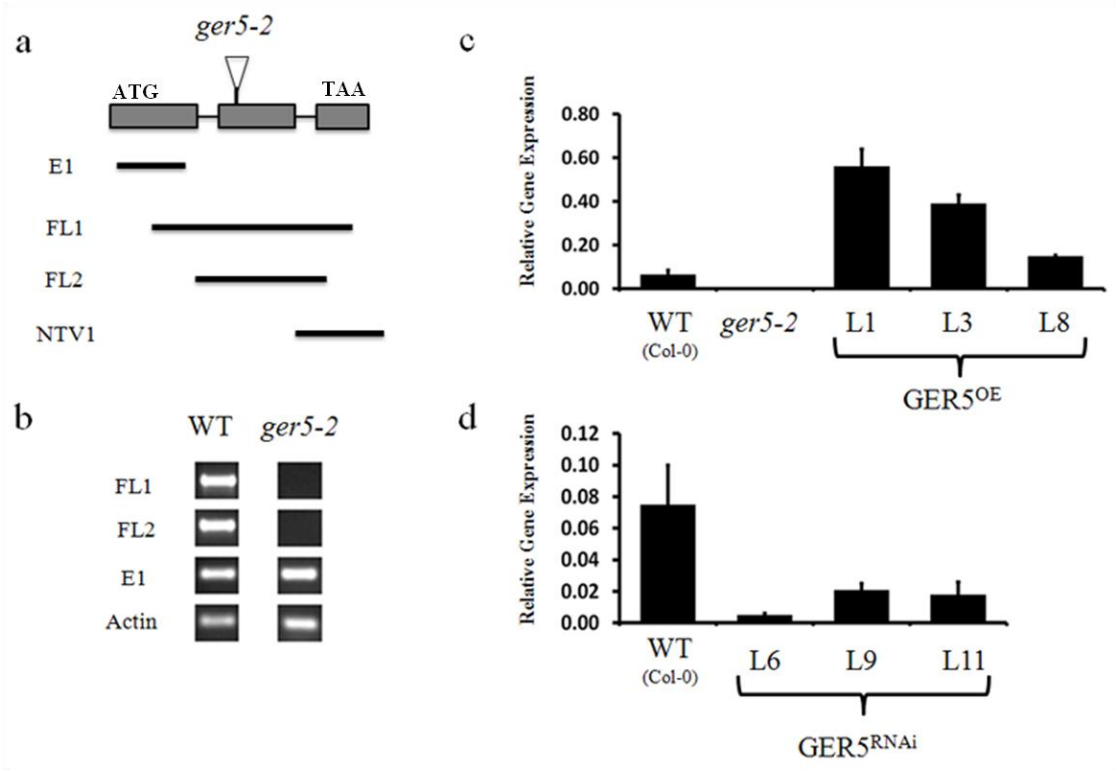

Fig. 3.1 Molecular characterization of *ger5-2* mutant and transgenic lines. **a** Intron-exon structure of *GER5* gene, location of T-DNA insertion, and positioning of primers in PCR and RT-qPCR studies. **b** *GER5*-derived transcripts in *ger5-2* mutant and wild-type plants. FL1 and FL2 primer sets amplify full-length *GER5* transcripts spanning T-DNA insertion site. E1 primers amplify exon located 5' of T-DNA insertion. **c** Relative levels of *GER5* expression in wild-type, *ger5-2* mutant and $GER5^{OE}$ lines as determined by RT-qPCR using FL2 primer set. **d** Relative levels of full-length *GER5* expression (native) in wild-type and $GER5^{RNAi}$ lines using NTV1 primer set. For PCR and RT-qPCR experiments cDNAs were derived from RNA extracted from pooled cauline leaves (n=6 plants per line). RT-qPCR data normalized to expression of internal reference gene *PP2A* (At1g13320). Data correspond to mean and standard deviation (SD) of triplicate reactions.

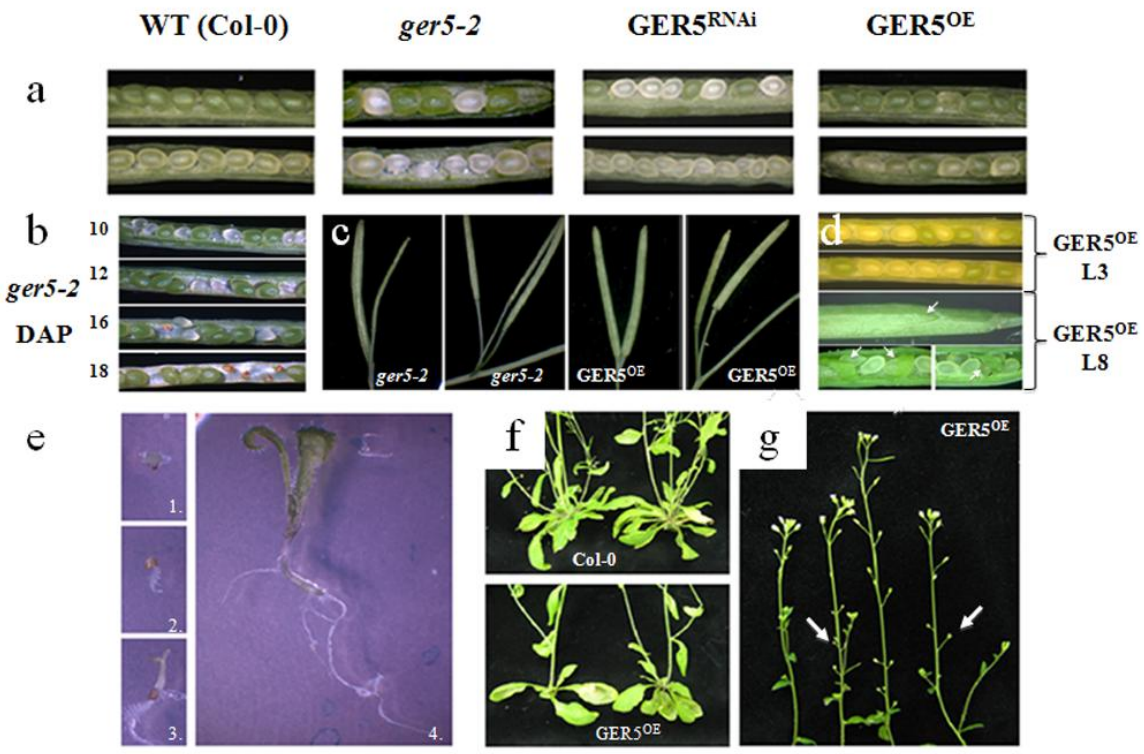

Fig. 3.2 Phenotype of *ger5-2* mutant, *GER5*^{RNAi} and *GER5*^{OE} lines. **a** Top (12-14 DAP) and bottom panels (8-12 DAP) depict siliques from identical plants but at different stages of development. **b** Translucent ovules within siliques of *ger5-2* mutants or *GER5*^{RNAi} lines (not shown) undergo senescence and abortion with progressive stages of reproductive development. DAP = days after pollination. **c** Seed abortion phenotypes of *ger5-2* mutant, *GER5*^{RNAi} lines (not shown) or *GER5*^{OE} line coincides with defects in silique morphology or patterning. **d** Developing seeds of *GER5*^{OE} lines can produce albino embryos, supernumerary carpels or leaf-like structures in place of ovules (white arrows). **e** Aborting seeds of *ger5-2* or *GER5*^{RNAi} lines can be excised and rescued by *in vitro* ovule culture. **f** For select *GER5*^{OE} lines, abortion of axillary meristems and alterations in inflorescence architecture accompanied seed development defects. **g** Floral abortion following emergence of inflorescence meristem in *GER5*^{OE} lines.

These phenotypes exhibited incomplete and variable penetrance, such that even in homozygous backgrounds only a proportion (27-64%, n ≥ 15 plants per line) of lines would display seed abortion or seed development phenotypes. Within siliques of *ger5-2* mutant and *GER5*^{RNAi} lines, visibly abnormal ovules were translucent and would ultimately abort and senesce with maturation of siliques (Fig. 3.2a,b, Table 3.1). At this stage of development an established *in vitro* ovule culture technique was utilized to obtain viable plants from seeds that would ultimately undergo abortion (Fig. 3.2b,e; Fig 3.2S) (Sauer and Friml 2004). In contrast to translucent ovules observed in *ger5-2* and *GER5*^{RNAi} lines, *GER5*^{OE} lines would generate opaque seeds containing green or albino embryos that were noticeably delayed relative to adjacent seeds in the same silique (Fig. 3.2a,d). In both loss-of-function and overexpression lines, seed abortion and seed

Table 3.1 Phenotypic characterization of mutant and transgenic lines grown under normal conditions.

Line	Plant Height	Axillary Branches	Silique Length	Seed Set (half silique)	Abnormal/Defective Ovules
	cm	*per plant*	*mm*		*% per silique*
Wild-type	**41.7 ± 0.6**	**6.6 ± 0.3**	**15.3 ± 0.4**	**31.6 ± 3.2**	**1.0 ± 0.4**
ger5-2	**41.2 ± 0.8**	**6.2 ± 0.2**	**15.1 ± 0.3**	**30.3 ± 2.9**	**40.6 ± 3.2***
***GER5*^{RNAi} (L11)**	**40.5± 0.5**	**6.5 ± 0.4**	**14.9 ± 0.4**	**30.9 ± 2.2**	**35.4 ± 2.9***
***GER5*^{OE} (L8)**	**34.2 ± 0.8***	**2.3 ± 0.3***	**11.4 ± 0.3***	**24.7 ± 2.7***	**47.8 ± 5.1***

Wild-type, *ger5-2* mutant, *GER5*^{RNAi} (L11) knockdown and *GER5*^{OE}(L8) overexpression lines were grown in parallel and assessed for plant height and axillary branching 48 days after germination (n ≥ 15 plants per line). Silique length and seed set (healthy + abnormal seeds) measurements were derived from plants displaying incomplete penetrance for seed abortion/development phenotypes (n ≥ 40 siliques per line). Data presented are means ± SE. *, Significant at P< 0.05 (Student's *t* test, as compared with the wild-type value).

development defects frequently coincided with defects in silique morphology and phyllotaxy (Fig. 3.2c; Table 3.1; Fig. 3.1S). For a fraction of *GER5*^{OE} lines defects in seed development coincided with production of supernumerary carpels, low levels of ovule abortion and formation of leaf-life structures in place of viable ovules (Fig. 3.2d; Fig. 3.1S). Relative to the wild type, loss-of-function lines showed no significant difference in plant height, axillary branching or total seed set; however, overexpression of *GER5* caused reductions in plant height, number of axillary branches per plant, silique length and total seed set (Table 3.1). These unique reproductive phenotypes seen in *GER5*^{OE} lines also coincided with abortion of axillary meristems and flowers (Fig. 3.2f,g) in addition to veinal chlorosis of rosette leaves (Fig. 3.1S). Taken together, our phenotypic analysis of loss- or gain-of-function lines indicates *GER5* participates in multiple aspects of reproductive development in plants. Recently, Choi and Hwang (2011) analyzed loss-of-function mutants associated with At5g13200 (*GER5*) and found seedlings to be compromised in pathogen response and ABA-salicylic acid antagonism.

3.4.2 Effects of *GER5* on embryo and seed development

To better understand the role of *GER5* during seed development, visibly defective ovules and adjacent healthy seeds were simultaneously extracted from siliques of mutant and transgenic lines for general histological study (Fig. 3.3a-n). Additional seed development defects (mild to severe) are shown in Fig. 3.3S1 through Fig. 3.3S3. For both *ger5-2* mutant and *GER5*^{RNAi} lines, embryos within white translucent seeds frequently arrested at the globular stage of development (Fig. 3.3a,b,f). Within these same ovules embryo arrest often accompanied proliferation of the chalazal endosperm (Fig. 3.3f). In rare instances *GER5*^{RNAi} produced seeds lacking embryos where

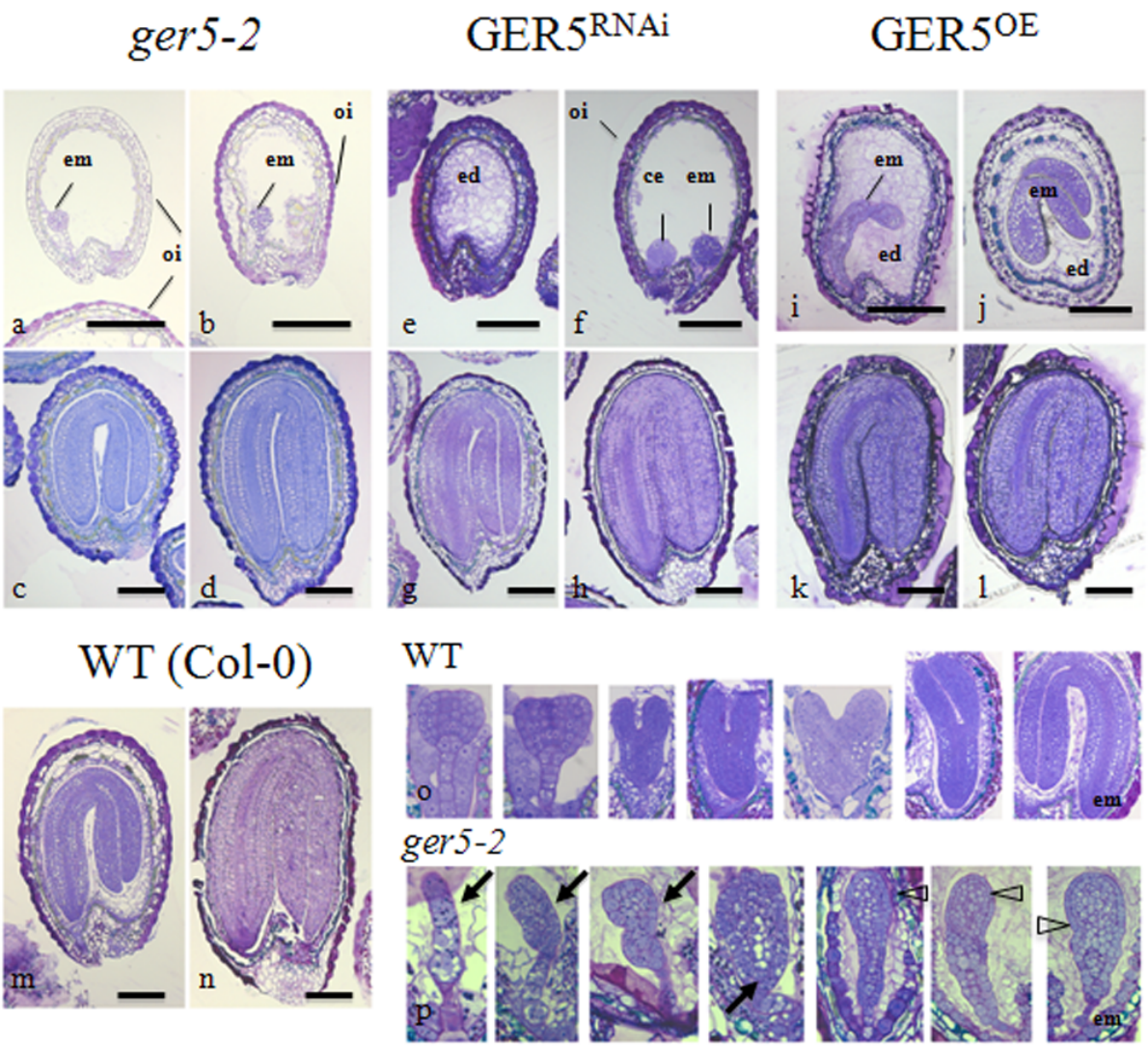

Fig. 3.3 Seed development and embryo defects of *ger5-2* mutant and transgenic lines. Longitudinal sections through *ger5-2* mutant (**a-d**), *GER5*^{RNAi}(**e-h**) *GER5*^{OE} (**i-l**) and wild-type seeds (**m,n**). For mutant and transgenic lines (**a-l**) bottom panels = healthy seeds, top panels = abnormal seeds. **o** Proper embryo development in wild-type seeds (globular, heart, torpedo, curled cotyledon, walking-stick). **p** Embryo defects in abnormal ovules extracted from *ger5-2* mutants. Black arrow mark disorganized embryo proper. Open arrowheads identify protoderm. em=embryo, oi=outer integument, ed=cellularized endosperm, ce=chalazal endosperm. Bar = 100 μm

proliferation and cellularization of the endosperm occurred (Fig. 3.3e). Across transluscent *ger5-2* and *GER5*^{RNAi} seeds, where embryo arrest uniformly occurred at the globular stage of development, considerable variation in differentiation of outer integuments was observed (Fig. 3.3a,b,f). At a gross morphological level *GER5*^{OE} seeds were not translucent and embryos appeared to lack pigmentation (Fig. 3.1a,d). Relative to *ger5-2* mutant and *GER5*^{RNAi} lines, arrest of embryos within defective seeds of *GER5*^{OE} lines consistently occurred at more advanced stages of embryo development and always coincided with endosperm cellularization (Fig. 3.3i,j). Taken together, these results suggest loss-and gain-of-*GER5* function triggers similar albeit unique perturbations in seed development. However, as homozygous loss-of-function mutants (*ger5-2*) are not embryo-lethal, *GER5* by itself does not appear to be essential for embryogenesis. Our histological studies also revealed a range of embryo defects within abnormal ovules extracted from mutant and transgenic lines (Fig. 3.3p; Fig. 3.3S). In wild-type seeds, embryogenesis proceeds sequentially through globular, transition, heart, torpedo and walking-stick stages of development (Fig. 3.3o, left to right). For visibly defective ovules extracted from *ger5-2* mutant and *GER5*^{RNAi} lines, embryos underwent asymmetric cell divisions following fertilization (Fig. 3.3p). However, at the proembryo stage of development, a proportion of embryos displayed aberrant morphology and disorganization of the embryo proper (Fig. 3.3p, black arrows). An even smaller proportion of embryos progressed to the globular stage of development and for this grouping, cell layers corresponding to ground tissue and vascular cylinder precursors appeared disorganized, whereas cell layers corresponding to the protoderm were clearly

defined (Fig. 3.3p, open arrowheads). Similarly, for *GER5*^{OE} lines, severely abnormal embryos were characterized by an intact protoderm and lack of internal organization (Fig. 3.3i; Fig. 3.3S). Collectively, these observations indicate *GER5* may participate in aspects of pattern formation during early stages of embryogenesis.

3.4.3 RNA *in situ* hybridization and ABA immunolocalization

To further elucidate sites of *GER5* action relative to reproductive and embryonic phenotypes observed in mutant and transgenic lines, RNA *in situ* studies were conducted on reproductive tissues of wild-type plants to assess qualitative patterns of *GER5* localization (Fig. 3.4a-i). During early stages of flower development preceding fertilization, *GER5* transcripts localized to both male and female reproductive organs (Fig. 3.4a). Within female reproductive organs, *GER5*-specific signals were detected throughout undifferentiated ovule primordia (Fig. 3.4a,b). However, with continued ovule development, *GER5* transcripts became restricted to ovule integuments (Fig. 3.4c). At early stages of flowering preceding the differentiation of male and female reproductive organs, *GER5* signals also displayed preferential localization within inflorescence meristems, floral meristems and floral organ primordia (Fig. 3.4d,g,h). Within male reproductive organs, *GER5*-specific signals also localized to anther primordia (Fig. 3.4e) and anther locules (Fig. 3.4a,d), in addition to pollen grains prior to dehiscence (Fig. 3.4f). Overall, RNA *in situ* studies revealed *GER5* transcripts were detected in reproductive tissues of Arabidopsis, but would preferentially localize in undifferentiated, meristematic cells of male and female reproductive organs.

In assessing *GER5* as a putative ABA-response gene we noted spatial patterns of *GER5* transcript accumulation (Fig. 3.4a-i) closely overlapped with previous reports of

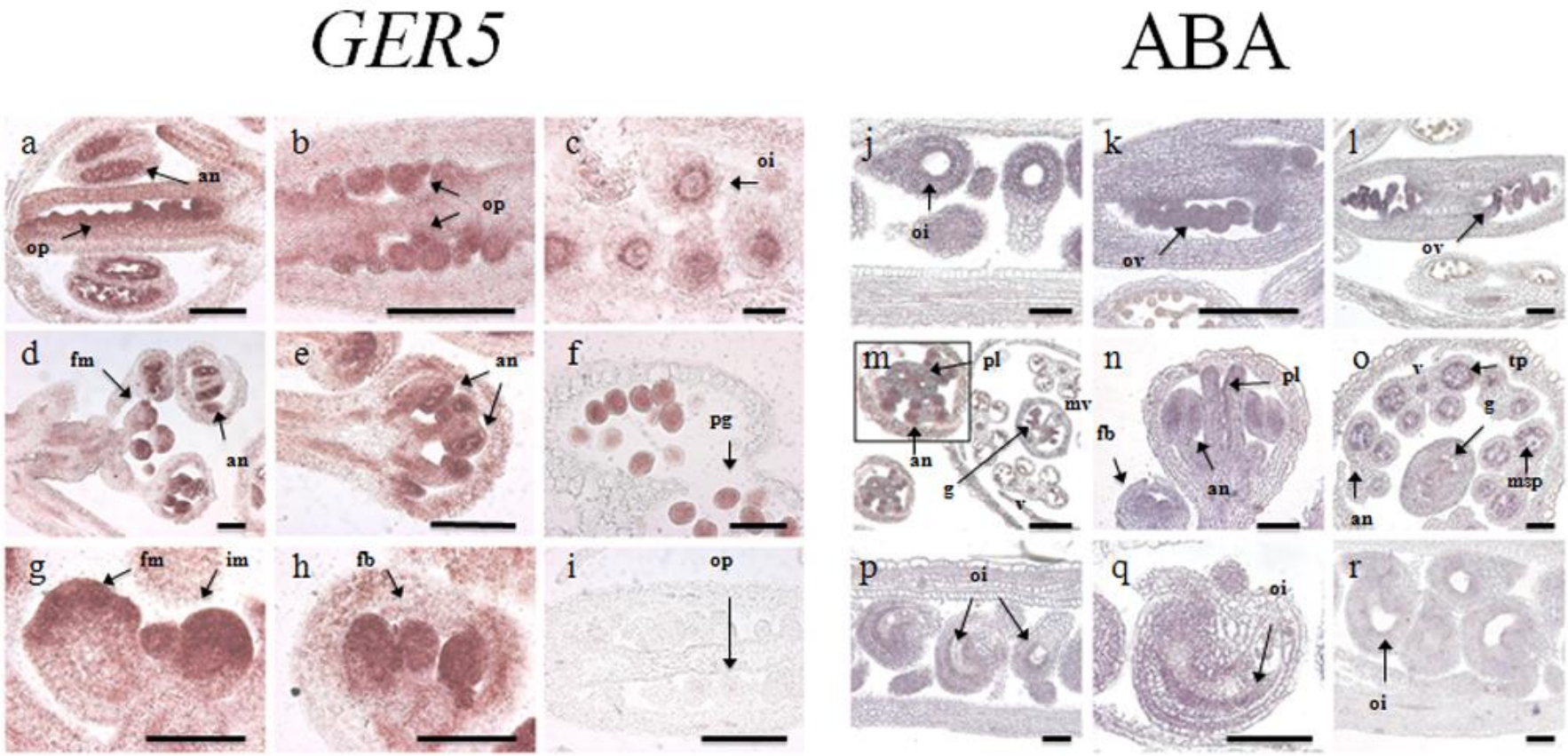

Fig. 3.4 *GER5* expression domain and abscisic acid (ABA) localization in reproductive organs of *Arabidopsis thaliana*. *GER5* expression (**a-i**), ABA localization (**j-r**). **a** Longitudinal section through floral bud (late stage 8 flower). *GER5* expression detected throughout locule of anther (an) and ovule primordia (op). **b** Longitudinal section through gynoecium (late stage 10 flower). *GER5* localizes to ovule primordia (op). **c** Longitudinal section through gynoecium (stage 11 flower). *GER5* localization is specific to ovule integuments (oi). **d** Longitudinal section through inflorescence meristems containing flowers at various stages of development (Stage 1-8). *GER5*-specific signals detected within floral meristems (fm) and anthers (an). **e** Longitudinal section through floral bud (stage 5 flower). *GER5* expression observed within central region of

Fig. 3.4 (continued**)** anthers and anther primordia **f** *GER5* expression in pollen grains (pg) (stage 12 flower). **g** and **h** *GER5* mRNA detected in infloresence meristem (im), floral meristem (fm) and floral bud. **i** Control hybridization with *GER5* sense probe. **j** Transverse section of unfertilized ovule (stage 12 flower). ABA localizes to ovule integuments (oi). **k** and **l** In stage 12 flowers ABA localizes to ovules (ov) and ovule funiculus of female gametophyte. **m** Transverse section through wild-type flowers (unstressed). Flower (stage 8) in lower left of panel has been magnified and inset. Within stage 8 flower, ABA localizes throughout entire anther (an), in addition to placenta (pl) of gynoecium. For stage 11 flower, ABA localizes specifically to vascular (v) region and microspores. ABA also localizes to ovules, funiculus and medial vascular bundle (mv) within gynoecium (g). **n** Longitudinal section through wild-type floral bud (stage 4, lower left) and developing flower (stage 8, middle). ABA localizes to organ primordia within floral bud (fb), central region of developing anthers (an), and placenta (pl) of developing gynoecium. **o** Transverse section through flower exposed to cold stress. ABA was detected in developing anthers (an) and gynoecium (g). ABA localizes prominently to tapetum (tp), developing microspores (msp) in addition to vascular region (v). **p** and **q** Longitudinal sections through unfertilized pistils, demonstrating ABA preferentially localizes to ovule integuments (oi). **r** Longitudinal section through pistil, showing negative control lacking primary antibody. Flower staging corresponds to Sanders et al. (1999).

ABA accumulation in reproductive organs of *A. thaliana* (Peng et al. 2006). This observation prompted us to localize ABA in our system (Fig. 3.4j-r). Similar to the expression domain of *GER5* in female reproductive organs, strong ABA-specific signals were detected in ovule integuments (Fig. 3.4j) and ovule primordia (Fig. 3.4j,k) prior to fertilization. Throughout flower development, ABA accumulated in both male and female reproductive organs (Fig. 3.4m,n,o). During early flower development ABA localization was concentrated in floral buds, the central region of anther locules, in addition to cell layers corresponding to the placenta (Fig. 3.4. m(inset), n). At later stages of flower development ABA accumulated preferentially in specific cells (e.g. funiculus, medial vascular bundle) of the developing gynoecium (Fig. 3.4 m,o). With continued stamen development, patterns of ABA localization became restricted to the tapetum,

developing microspores and vascular tissues (Fig. 3.4m,o). During later stages of ovule development, ABA localization was highly concentrated in both inner and outer integuments of female gametophytes (Fig. 3.4p,q). For plants exposed to cold stress (0°C) strong ABA signals were detected in the tapetum, microspores and vascular region of the flower (Fig. 3. 4o). In contrast, no changes in ABA localization patterns were observed between plants subjected to heat stress (37°C) and unstressed controls grown at 22°C (data not shown). RNA *in situ* and ABA localization studies revealed that in the absence of stress, *GER5*-transcripts localize to early reproductive tissues which preferentially accumulate ABA. ABA-related phenotypes associated with *ger5-2* mutants (see following sections) also led us to evaluate the expression domain of *GER5* during seed development (Fig. 3.6S). The uniform distribution of *GER5* in developing embryos and aleurone layer (Fig. 3.6S) suggested a broad expression of *GER5* during both pre-and post-fertilization events. In addressing potential issues of functional redundancy between *GER5* and closely related members of the GRAM domain family (see following sections), *GER1* and *GEM* transcripts were also localized in reproductive organs of *A. thaliana* (Fig. 3.6S). Relative to *GER5*, *GER1* and *GEM* displayed unique expression domains in these same reproductive organs. For example, *GER1* transcripts preferentially localized to the silique wall, whereas *GEM* transcripts localized to the stigma and transmitting tract (Fig. 3.6S). However, for a number of tissues (e.g. inflorescence meristems, ovule primordia, ovule integuments) we noted *GER1* or *GEM* expression domains would overlap with those of *GER5* (Fig. 3.4a-i; Fig. 3.6S).

3.4.4 Expression analysis of *GER5*, *GER1* and *GEM* in reproductive tissues

As multiple members of the GRAM family are characterized as putative ABA-response genes (Jiang et al. 2008) and *in silico* analysis of promoters revealed the presence of *cis*-response elements associated with ABA-dependent and ABA-independent signaling pathways, we sought to evaluate how hormones and abiotic stress treatments influenced *GER5*, *GER1* and *GEM* expression in reproductive organs. GRAM gene expression was also assessed across various tissues (seedlings, mature seeds) of wild-type plants (Col-0), in addition to reproductive organs of mutants (*aba1, aba2, ctr1*) defective in hormone biosynthesis or sugar signaling. Across tissues isolated from vegetative seedlings (VM, VL, VR), reproductive stage plants (CL, IM, DS) and mature seeds (MS) (Fig. 3.5), expression of GRAM genes was examined (Fig. 3.5a,b,c). For *GER5*, the lowest relative levels of gene expression were detected in mature, dormant seeds (Fig. 3.5a). For *GER1* and *GEM*, relative expression levels were consistently lower in reproductive tissues and mature seeds when compared to tissues isolated from seedlings (Fig. 3.5b,c). Overall, these results suggest *GER5*, *GER1* and *GEM* are broadly expressed during plant development. In cauline leaves, expression of all three genes increased following cold stress (Fig. 3.5d,e,f). However, within inflorescence meristems and developing siliques, only *GER1* expression increased in response to cold stress (Fig. 3.5 h,k), whereas *GER5* and *GER1* expression was slightly repressed by heat stress in cauline leaf and developing silique (Fig. 3.5 e,j,k). Overall, these GRAM genes were differentially expressed (both up- and down-regulated) depending upon the organ and stress evaluated. In response to exogenous applications of ABA, transcript levels of

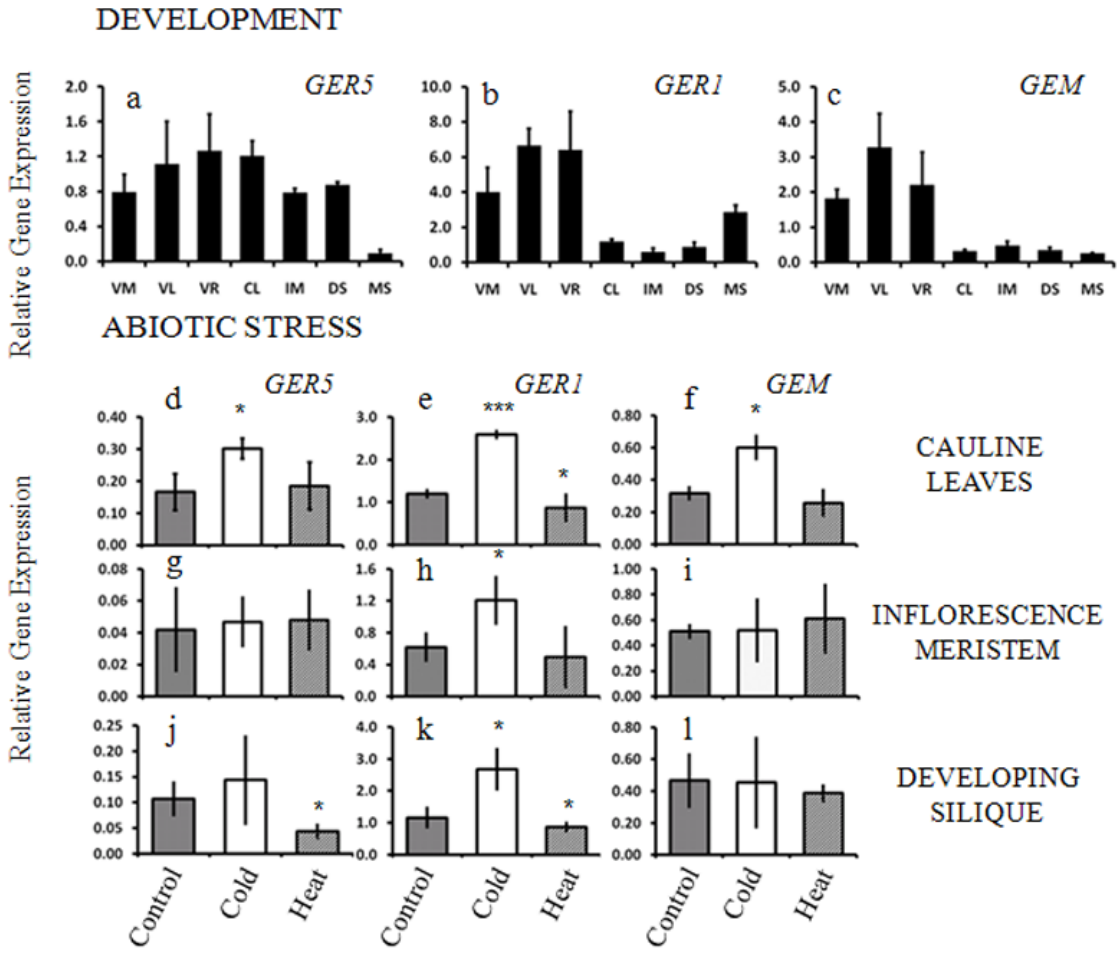

Fig. 3.5 Expression of GRAM genes during development and following environmental stress. **a** *GER5*, **b** *GER1*, **c** *GEM* expression during plant development. **d-l** Expression of *GER5*, *GER1*, *GEM* in reproductive organs exposed to cold and heat stress. RT-qPCR data normalized to reference gene *PP2A* (*At1g13320*). Asterisks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to plants at 22°C. Data correspond to mean and standard deviation (SD) of three biological replicates. VM = seedling meristem, VL = seedling leaves, VR = seedling roots, CL = cauline leaves, IM = inflorescence meristem, DS = developing silique, MS = mature seed.

all three GRAM genes increased within inflorescences (reproductive tissues combined) (Fig.3.6a-c). In the current study, GA treatments did not alter the expression of any GRAM gene assessed (Fig. 3.6a-c). Surprisingly, in cauline leaves of ABA-deficient mutants (*aba1, aba2*), *GER5* expression was elevated relative to wild-type plants grown in parallel (Fig. 3.6d). However, within inflorescence meristems of these same plants, *GER5* expression was repressed (Fig. 3.6g). Moreover, while *GER5* expression increased in cauline leaves of ABA-deficient mutants, *GER1* expression was reduced (Fig. 3.6e). Within the *ctr1* mutants of *A. thaliana*, which display constitutive ethylene responses that antagonize ABA metabolism and signaling, *GER5*, *GER1* and *GEM* expression was up- or down-regulated in a tissue-specific manner (Fig. 3.6e,g,i,j). For developing siliques, *GER5* transcripts were unaltered in ABA-deficient backgrounds (*aba1, aba2*) but were up-regulated in *ctr1* mutants (Fig. 3.6j). In comparison to *GER1* and *GEM*, our results suggest *GER5* expression is more responsive to genetic lesions affecting hormone biosynthesis or signaling. Furthermore, where cold stress and exogenous ABA treatments led to widespread activation of GRAM genes, mutants deficient in ABA biosynthesis were characterized by complex developmental patterns of GRAM gene expression. Together, these results indicate members of the GRAM gene family may be differentially regulated in a cell-type or tissue-specific manner.

3.4.5 Seed germination assays

To further explore the relationship between ABA and *GER5*, germination assays were conducted upon age-matched seeds obtained from *ger5-2* mutants displaying the seed abortion phenotype in addition to plants derived from the same seed stock but not displaying the seed abortion phenotype. In both instances, *ger5-2* mutant seeds

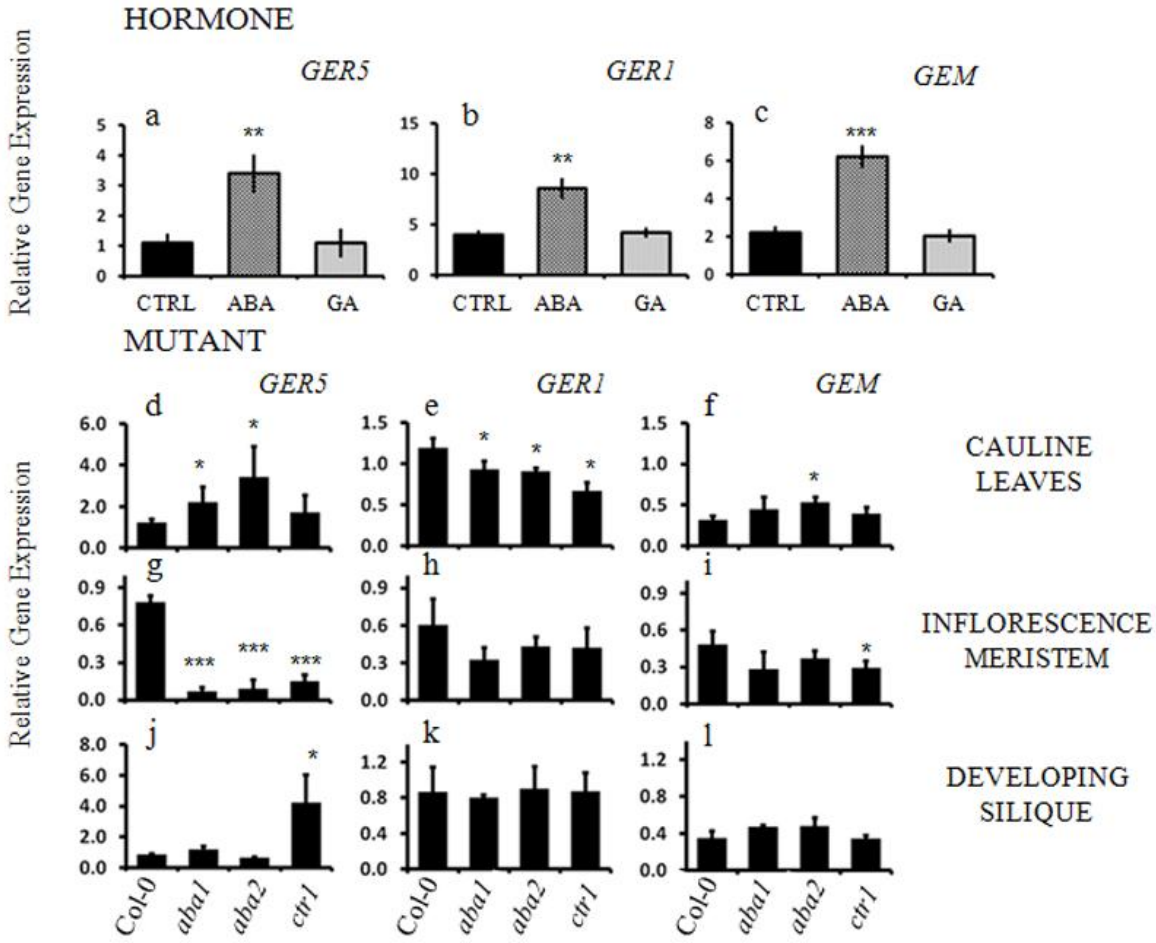

Fig. 3.6 GRAM gene expression in response to hormones or within mutants with defects in hormone biosynthesis and signaling. Expression of **a** *GER5*, **b** *GER1* and **c** *GEM* within complete inflorescences treated with ABA, GA or without hormones (control). **d-l** Expression of GRAM genes (*GER5*, *GER1*, *GEM*) in reproductive organs of WT plants and mutants deficient in ABA biosynthesis (*aba1*, *aba2*) or ethylene signaling (*ctr1*). RT-qPCR data are normalized to the expression of the internal reference gene *PP2A* (At1g13320). Asterisks ($P \leq 0.05$ = * or $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to control plants grown at 22°C. Note changes in relative expression scale between genes and reproductive organs. Data correspond to mean and standard deviation (SD) of three biological replicates.

performed similarly in ABA germination assays, which also incorporated *aba2-1* mutant seeds as a control. As shown in Fig. 3.7a, relative to wild-type seeds, *ger5-2* mutant seeds displayed significant increases in percent germination in the presence of exogenous ABA, but less than that observed for *aba2* mutants cultured on similar ABA-containing media (Fig. 3.7a). As an extension of time-course assays, germination of seed genotypes was evaluated in the presence of compounds affecting metabolism or signaling of ABA or GA (Fig. 3.7b). After 72 hours, no significant differences in germination were observed between seed genotypes cultured on control plates or media containing GA_3 (Fig. 3.7b). However, in the presence of paclobutrazol, a gibberellin biosynthesis inhibitor, % germination of *aba2* and *ger5-2* seeds was significantly higher that wild-type seeds (Fig. 3.7b). On media containing fluridone, an inhibitor of ABA biosynthesis, germination was not significantly different between seed genotypes. However, when ABA was applied with fluridone, germination of *ger5-2* mutants was significantly higher than wild-type seeds (Fig. 3.7b), indicating *ger5-2* mutant seeds appear to exhibit alterations in ABA sensitivity rather than ABA deficiency. In the presence of uniconazole-P, an inhibitor of ABA catabolism, wild-type and *ger5-2* mutant seeds did not germinate. However, *aba2* mutants displayed low rates of germination on media containing uniconazole-P (Fig. 3.7b). Taken together, seed germination assays provided evidence *ger5-2* mutants are characterized by reduced sensitivity to ABA (or hypersensitivity to GA) during seed development and seed germination.

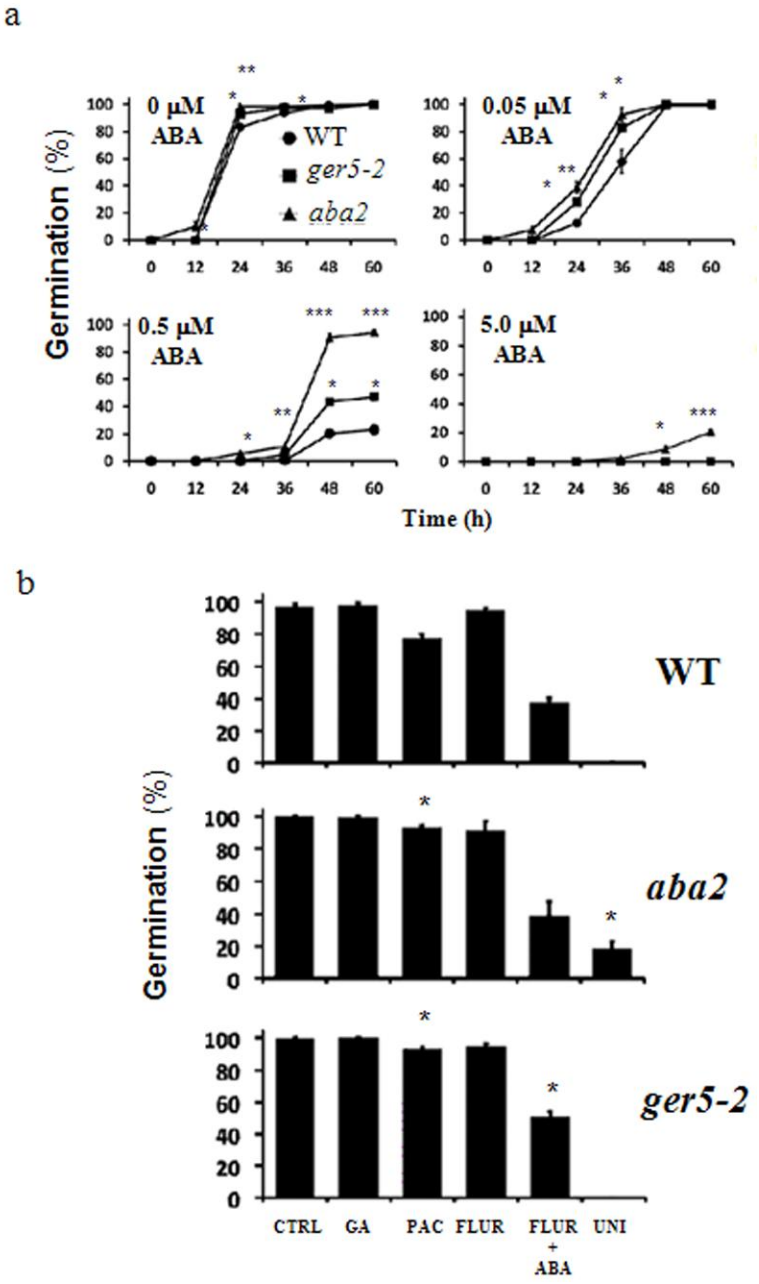

Fig. 3.7 Arabidopsis *ger5-2* mutants are less responsive to ABA during seed germination assays. **a** Percent seed germination of wild-type (Col-0), *ger5-2* mutant and *aba2-1* mutant as a function of time in the presence of 0.0, 0.05, 0.5 and 5.0 µM *cis*-ABA. **b** Percent seed germination of wild-type (Col-0), *ger5-2* mutant and *aba2-1* mutant after 72 h. CTRL = control, GA = gibberellic acid (5 µM), PAC = paclobutrazol (5 µM), FLUR = fluridone (2.5µM), ABA = abscisic acid (2.5µM), UNI = uniconazole (100 nM). Asterisks indicate significant differences from the Col-0 control. (*P<0.05, **P<0.01, ***P<0.001) as determined by two-tailed student's t-test. Data correspond to mean and standard error (SE) of a single experiment repeated in triplicate.

3.4.6 Genome-wide analysis of seed abortion and seed development in *ger5-2* mutants

To further elucidate molecular pathways contributing towards seed development defects and altered ABA response of *ger5-2* mutants, microarray analysis was undertaken to contrast the transcriptional profile of wild-type seeds (WT) against green (HG) and white (HW) seeds isolated from homozygous *ger5-2* mutants (Fig. 3.8a). In analysis scenario 1 (fold change ≥ 1.25, FDR ≤ 0.05) 656 oligonucleotide probe sets were differentially expressed across comparisons of seed categories, with the majority of probe sets associated with direct comparisons between white seeds (HW) about to undergo abortion and green seeds isolated from wild-type (WT) or *ger5-2* mutant (HG) plants (Fig. 3.8b). This observation was consistent with visual differences in anatomy or physiological status of HW seeds relative to HG or WT seeds. Additional probe sets (n= 108) differentially expressed between WT and HG seeds were identified in an alternative analysis scenario (fold change ≥ 1.1, FDR ≤ 0.20) (Fig. 3.8b, Appendix 8.1). Ultimately, genes identified under both analysis scenarios in the microarray study were selected and subsequently validated by RT-qPCR (Fig. 3.7S).

Based on functional annotation of differentially expressed probe sets (n = 763, Fig. 3.8b) using Virtual Plant 1.2 (Katari et al. 2010), major functional categories included genes involved in cellular (27.4%) and metabolic (21.5%) processes, biological regulation (12.8%), and response to stimulus (13.3%) (Fig. 3.8c). Amongst major categories, genes involved in carbohydrate metabolism and cell wall modification displayed the greatest fold-change in expression (Table 3.2). Within this category, several classes of carbohydrate active enzymes (e.g. cell wall invertases, invertase inhibitor

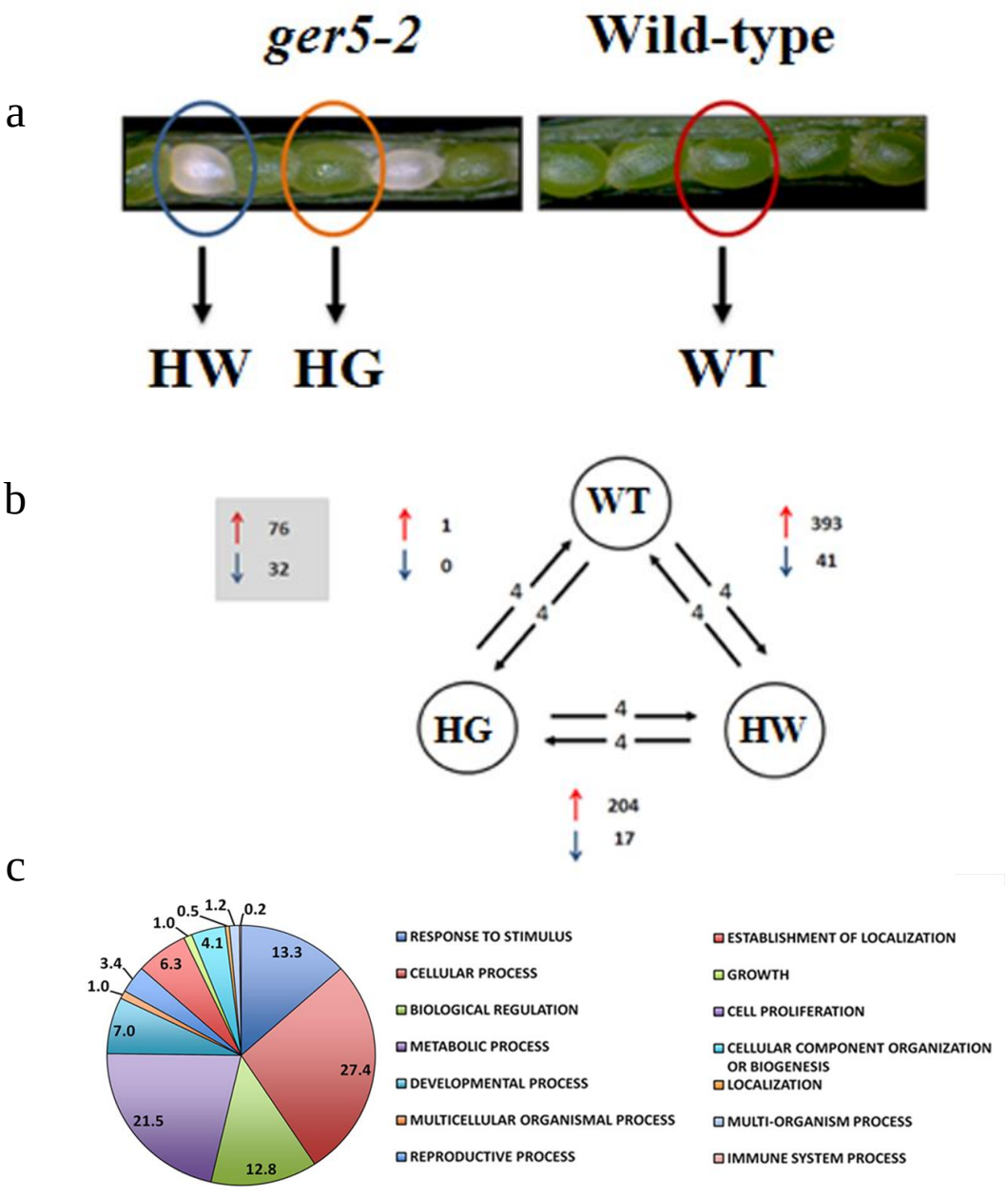

Fig. 3.8 a Diagram of seed categories (HW, HG, WT) analyzed for transcriptional profiling in microarray experiments. **b** Experimental design of microarray experiment. For each seed category four (4) biological replicates (consisting of 50-70 extracted seeds) were reversed labeled with Cy3 and Cy5 dyes. In total 24 labelled aRNA were hybridized to 12 microarray slides. For direct comparisons up- and down-regulated probe sets are indicated by red and blue arrows, respectively. Up- and down-regulated probe sets shaded in grey were identified in analysis scenario 2. **c** Functional classification of genes differentially regulated between seed categories. Pie charts represent 763 probes showing differential expression.

proteins, glucosyltransferases) were differentially expressed between seed categories, indicating seed development defects observed in *ger5-2* mutants could be correlated with changes in carbohydrate status of seeds.

Numerous genes associated with carbohydrate metabolism, cell wall modification, autophagy and protein degradation were also differentially expressed between seed categories (Table 3.2). Notably, four of nine serine carboxypeptidase genes (At1g73270, At1g73290, At1g73300, At1g73310) which occur in tandem on chromosome 1 were coordinately down-regulated in HW seeds (Table 3.2). Two of these family members (At1g73300, At1g73310) and a separate serine carboxypeptidase (At2g23010) were up-regulated in HG seeds relative to WT seeds (Table 3.2). Several arabinogalactan-related proteins (AGP), which are highly glycosylated and typically associate with the cell wall or apoplastic space of plant cells, were also differentially expressed amongst seed categories (Table 3.2).

Beyond changes in carbohydrate metabolism or cell wall modification, seed development defects of HW seeds were accompanied by transcriptional differences in hormone signaling. Several members of the gibberellic acid-stimulated Arabidopsis (GASA) gene family (*GASA1, 3, 4* and *5*) were differentially regulated amongst seed categories (Table 3.3). *GASA* genes tend to be highly expressed within meristematic regions and encode small polypeptides that may be secreted into the extracellular space of plant cells (Roxrud et al. 2007). In addition to GASA family members, several genes involved in GA metabolism (*GA20ox, GGPS1*) or signaling (*RGL1*) were also up- or down-regulated in direct comparisons between seed categories (Table 3.3). Several characterized ABA-response genes were also differentially expressed across seed

Table 3.2 Summary of differentially expressed genes associated with carbohydrate metabolism and autophagy

Accession AGI	Gene Description	Microarray Fold-Change WT/HW	Microarray Fold-Change HG/HW	Microarray Fold-Change WT/HG	Validation by RT-qPCR
Carbohydrate Metabolism, Cell Wall Modification					
At4g02250	invertase/pectin methyesterase inhibitor	6.4	7.4		**
At3g17130	invertase/pectin methyesterase inhibitor	3.2	5.8		
At4g15750	invertase/pectin methyesterase inhibitor	**-18.3**	**-38.0**		**
At1g62760	invertase/pectin methylesterase inhibitor			1.3	**
At3g13790	cell wall invertase, beta-fructosidase	6.1	5.2		
At3g43270	pectinesterase family protein	4.3	5.7		
At5g20860	pectinesterase family protein	3.8	5.7		
At4g15210	beta-amylase (BMY1)	**-8.8**	**-14.3**		
At5g20560	beta-1,3-glucanase	4.9	6.4		
At2g01850	xyloglucan:xyloglucosyl transferase	**-3.5**	**-4.3**		
At3g48580	xyloglucan:xyloglucosyl transferase	**-6.5**	**-10.8**		
At3g10740	glycosyl hydrolase family 51 protein	**-2.8**	**-2.1**	1.4	
At2g16230	glycosyl hydrolase family 17 protein		5.2	**-1.9**	
At1g22400	UDP-glucoronosyl/UDP-glucosyl transferase	2.2	5.0	**-2.3**	**
At3g46680	UDP-glucoronosyl/UDP-glucosyl transferase	10.3	15.6		
Autophagy Pathway					
At1g73270	serine carboxypeptidase S10 family protein	3.9	4.8		
At1g73290	serine carboxypeptidase S10 family protein	5.3	8.6		
At1g73300	serine carboxypeptidase S10 family protein	2.1	3.0	**-1.4**	
At1g73310	serine carboxypeptidase S10 family protein	2.3	5.2	**-2.2**	**
At2g23010	serine carboxypeptidase S10 family protein	2.4	4.2	**-1.9**	
At4g12910	serine carboxypeptidase-like 20	**-6.1**	**-6.9**		
At5g08260	serine carboxypeptidase-like 35	**-4.9**	**-5.3**		
At3g12203	serine carboxypeptidase-like 17	6.9	7.4		
At2g21430	cysteine proteinase A494	**-4.2**	**-6.8**		**
At3g54940	cysteine proteinase	3.8	3.4		**
At1g62710	vacuolar processing enzyme beta / beta-VPE	2.7	2.5		
At4g30030	aspartyl protease	3.4	4.3		

Values shown are fold changes for corresponding direct comparisions between genotypes and phenotypes (WT/HW, HG/HW, WT/HG)
Regular text indicates genes up-regulated; boldface text indicates genes down-regulated
Fold-change values not highlighted identified in scenario 1 (FDR ≤ 0.05, 1.25-fold change in gene expression)
Fold-change values highlighted in grey identified in scenario 2 (FDR ≤ 0.20, 1.1-fold change in gene expression)

Table 3.3 Summary of differentially expressed genes associated with hormone metabolism and signaling

Accession AGI	Gene Description	Microarray Fold-Change WT/HW	Microarray Fold-Change HG/HW	Microarray Fold-Change WT/HG	Validation by RT-qPCR
Gibberellin biosynthesis and response					
At1g75750	gibberellin-regulated protein (GASA1)	**-5.3**	**-5.2**		**
At2g14900	gibberellin-regulated protein (GASA4)	**-4.2**	**-6.7**		
At5g14920	gibberellin-regulated family protein	2.4	2.2		**
At4g09600	gibberellin-regulated protein 3 (GASA3)		29.3		
At2g30810	gibberellin-regulated (similar to GASA5)		**-1.4**		
At1g74670	gibberellin-responsive protein		2.1		
At1g66350	gibberellin regulatory protein (RGL1)		**-2.7**		
At3g29420	geranylgeranyl pyrophosphate	6.3	7.6		
At5g51810	gibberellin 20-oxidase	1.9	2.5		
Abscisic acid biosynthesis and response					
At3g24650	abscisic acid-insensitive protein 3 (ABI3)	2.5	2.5		
At2g40220	abscisic acid-insensitive 4 (ABI4)	2.2	2.5		
At5g15960	stress-responsive protein (KIN1)	8.9	11.8		
At5g15970	stress-responsive protein (KIN2) (COR6.6)		7.5		
At3g02480	ABA-responsive (similar to KIN1)	2.6	3.4		
At2g42820	abscisic acid-responsive HVA22 family protein	3.0	4.1		**
At5g62490	ABA-responsive protein (HVA22b)		3.5		
At4g36720	abscisic acid-responsive HVA22 family protein	1.2	1.2		
At5g08350	GRAM domain-containing protein	**-1.8**	**-2.0**		
At5g50170	C2 domain/ GRAM domain-containing protein	3.6	7.4		
At2g22475	GRAM domain/ ABA-responsive protein-related		**-2.8**		
Arabinogalactans					
At5g40730	arabinogalactan-protein (AGP24)	**-4.8**	**-7.4**		
At4g31370	fasciclin-like arabinogalactan family protein	**-8.5**	**-14.7**		**
At4g40090	arabinogalactan-protein (AGP3)			1.2	
At2g22470	arabinogalactan-protein (AGP2)		**-1.2**	1.3	**

Values shown are fold changes for corresponding direct comparisions between genotypes and phenotypes (WT/HW, HG/HW, WT/HG)

Regular text indicates genes up-regulated; boldface text indicates genes down-regulated

Fold-change values not highlighted identified in scenario 1 (FDR ≤ 0.05, 1.25-fold change in gene expression)

Fold-change values highlighted in grey identified in scenario 2 (FDR ≤ 0.20, 1.1-fold change in gene expression)

categories (Table 3.3). Two well-characterized ABA and stress-responsive genes (*KIN1*, *KIN2*) located in tandem on chromosome 5, alongside a *KIN1*-like gene located on chromosome 3, were similarly expressed in direct comparisons between seed categories (Table 3.3). Alongside ABI3 and ABI4 transcription factors, multiple members of an ABA-responsive gene family (*AtHVA22*) associated with programmed cell death and/or autophagy were differentially expressed in HW seeds (Table 3.3) (Guo and Ho 2008; Chen et al. 2009). Several closely related members of the GRAM domain family (At5g08350, At5g50170, At2g22475) were differentially expressed between HG and HW categories (Table 3.3). However, amongst classes of ABA-response genes identified, members of the GRAM family were both up- and down- regulated in HW seeds. Overall, our microarray study revealed the ABA-insensitive phenotype of *ger5-2* mutant seeds could be correlated with specific transcriptional differences in genes associated with hormone metabolism and signaling.

3.5 Discussion

Forward and reverse genetic studies have led to the isolation of numerous loci involved in ABA responses. However, little information is currently available describing the function of ABA-responsive GRAM genes in plant growth, development and stress response. In the current study we sought to functionally characterize *GER5* (***GE****M-****R****ELATED* ***5****),* an *A. thaliana* homolog of barley *ABA45* and pepper *CaABR1* (Liu et al. 1999; Choi and Hwang 2011). During the course of assessing *GER5*, our characterization efforts expanded to include *GER1* and *GEM*, two related members of the GRAM gene family in *A. thaliana* (Jiang et al. 2008; Caro et al. 2007).

3.5.1 *GER5* affects reproductive development

In this work, *ger5-2* mutants and transgenic lines ($GER5^{OE}$, $GER5^{RNAi}$) were characterized by defects in reproductive development affecting seed development and inflorescence architecture (Fig. 3.2; Fig. 3.1S), indicating *GER5* plays a role in multiple facets of reproductive development in plants. RNA *in situ* studies further indicate *GER5* was expressed throughout inflorescence and floral meristems, male and female reproductive organs, and within zygotic and maternal tissues during seed development (Fig. 3.4a-i, Fig. 3.6S). Alongside gene expression analysis, RNA *in situ* studies revealed *GER5*, *GER1* and *GEM* transcripts had both unique and overlapping expression domains within reproductive organs. However, given the broad expression of all three genes during plant development, we cannot exclude potential roles for GRAM genes beyond reproductive development. For example, VAD1 and GER5/CaABR1 participate in the cell death response of leaves during plant-pathogen interactions (Lorrain et al. 2004; Choi and Hwang 2011) and GEM mediates patterning events during root development (Caro et al. 2007).

At a molecular level existing studies indicate GEM, through protein-protein interaction with cell division (CDT1) and patterning (TTG1) proteins, serves as a novel regulator of cell division, patterning and differentiation within epidermal cells of Arabidopsis roots and leaves (Caro et al. 2007). During fiber development within cotton (*Gossium* spp) seeds, Deng et al. (2012) also identified a PROTODERMAL FACTOR1 (GhPDF1) protein capable of interacting with a cotton homolog of GER5. In Arabidopsis, PDF1 encodes a putative extracellular protein that localizes exclusively to the L1 layer of shoot apices, protoderm of organ primordia, and has been implicated in

aspects of cell-fate determination of epidermal tissues (Abe et al. 2001). Several epidermal patterning proteins (TTG1, PDF1) or genes (*GL2, CPC*) that interact directly with or downstream of GRAM proteins also function in embryo development, seed coat differentiation and mucilage synthesis (Costa and Dolan 2003; Zhang et al. 2003). A regulatory subunit (PP1c) of protein phosphatase P1 (PP1) forming direct protein-protein interactions with two GRAM proteins (PRSL1, GEM) also functions during early stages of embryogenesis in addition to mediating cross-talk between blue light and ABA signaling pathways (Takemiya et al. 2012). Collectively, these studies indicate multiple members of the GRAM family interact with proteins involved in epidermal patterning, cell fate specification and embryogenesis, and provide supporting evidence GRAM domain proteins function in reproductive development. In the current study, reproductive phenotypes observed in mutant and transgenic lines (Fig. 3.2; Fig. 3.1S) suggest *GER5* may function in patterning-related events associated with inflorescence architecture and seed development. This observation is new and suggests future studies may seek to clarify the relationship between *GER5* and characterized proteins involved in epidermal patterning and cell fate specification.

RNA *in situ* studies showed *GER5, GER1* and *GEM* are broadly expressed in reproductive organs and developing seeds (Fig. 3.4; Fig 3.6S), indicating putative roles for GRAM genes in reproductive development that extend beyond cell cycle regulation and cell fate specification. Previously, Jiang et al. (2005) identified a C2-GRAM gene of rice that is expressed in a tissue-specific manner and contributes towards the pollen-less phenotype of *Oryza sativa no pollen* (*Osnop)* mutants. In barley, *ABA45* is also expressed in a tissue-specific manner, with transcripts accumulating to high levels in

developing embryo and aleurone tissues treated with ABA (Liu et al. 1999). Kakumanu et al. (2012) also reported that relative to basal leaf meristem tissues, numerous GRAM genes are up-regulated in maize (*Zea mays*) ovaries exposed to drought stress. Given results obtained in the current study, these observations suggest that across monocot and dicot species, GRAM domain genes may hold critical roles during the reproductive stage of plant development.

During the course of characterizing *GER5* as an ABA- and stress-responsive gene related to barley *ABA45* and *CaABR1* we noted the expression domain of *GER5* closely overlapped with patterns of ABA accumulation in reproductive organs of Arabidopsis (Peng et al. 2006). This observation prompted our own assessment of ABA localization in reproductive organs, in conjunction with a transcriptional analysis of ABA metabolism, transport and homeostasis in reproductive organs exposed to abiotic stress (Baron et al. 2012). Our transcriptional analysis of *GER5*, *GER1* and *GEM* in these same reproductive organs revealed complex developmental patterns of gene expression. For example, both cold stress and exogenous ABA treatments led to simultaneous up-regulation of GRAM genes in several organs (Fig. 3.5; Fig. 3.6). However, across ABA-deficient and ethylene signaling mutants, GRAM genes were up- or down-regulated in a tissue-specific manner (Fig. 3.6). Similar family-wide assessments of GRAM gene expression have revealed divergent expression patterns with plant development and environmental stress (Jiang et al. 2008; Kakumanu et al. 2012). Frank et al. (2009) also determined orthologs of *GER5* and *GEM* were induced or repressed, respectively, in tomato (*Solanum lycopersicum*) microspores exposed to heat stress. In our microarray and gene expression studies, GRAM genes were uniquely regulated relative to

characterized genes involved in ABA metabolism and response (Baron et al. 2012). Together, these observations suggest that the transcriptional regulation of GRAM genes during stress responses may differ significantly from their regulation during plant development.

Increasing evidence suggests ABA functions as a critical regulator of invertase activity and monosaccharide transporter gene expression in vegetative and reproductive organs (Ruan et al. 2010; Dolferus et al. 2011). During seed development INV and inhibitory proteins (INVIN) regulate the supply of hexoses to embryo and endosperm tissues, which undergo contrasting rates of cell division and development (Bate et al. 2004; Jin et al. 2009). Differences in activity or sub-cellular targeting of INV, pectin methylesterase (PME) or associated inhibitory proteins (INVIN/PMEI) also modify phyllotaxy, flowering time, inflorescence architecture and seed yield in Arabidopsis (Heyer et al. 2004; Peaucelle et al. 2008). In the current study, seed development defects of *ger5-2* mutants were accompanied by transcriptional changes in genes regulating INV or PME activities in seeds (Table 3.2). Alongside genes involved in hormone metabolism and signaling, many differentially expressed genes identified in the current study encode hydrolytic enzymes responsible for cleaving sucrose into glucose and fructose. Genes encoding enzymes (e.g. amylases, glycosyl hydrolase) involved in the hydrolysis of starch or degradation of cell wall polysaccharides were also differentially expressed amongst seed categories. These observations indicate the role of *GER5* in reproductive development and stress responses may be associated with aspects of sugar metabolism or assimilate transfer that contribute towards the maturation, secretion or even activation of carbohydrate active enzymes.

3.5.2 *GER5* modifies ABA responses during seed development and germination

In the current study seed germination assays provided evidence *ger5-2* mutants were less responsive to ABA, hypersensitive to GA, or capable of synthesizing greater quantities of GA (Fig. 3.7). Our microarray analysis further revealed several ABA and GA-regulated genes were differentially expressed when WT/HG seeds were contrasted with HW seeds. However, by comparing lists of differentially expressed genes we identified at least two characterized negative regulators of GA signaling (*RGL1, GASA5*) that were down-regulated in HG seeds, but were not differentially expressed in WT seeds (Wen and Chang 2002; Zhang et al. 2009). These observations provide supporting evidence the ABA-related phenotype of *ger5-2* mutants during seed germination assays could be correlated with transcriptional differences in hormone metabolism or signaling. Taken together, these results indicate *GER5* modifies ABA responses during seed dormancy and germination.

Several observations in the current study led us to speculate the activity of *GER5* and related GRAM genes may be associated with aspects of sugar signaling, sugar transport or carbohydrate metabolism. First, *in vitro* ovule culture medium containing high levels of sucrose (10%) facilitated the conversion of excised ovules (HW) to viable seedlings. As comparable ovules undergo abortion and senescence within siliques of *ger5-2* mutants, these observations suggest maternal deficiencies in sugar metabolism or delivery may underlie the abortion and senescence of ovules (*in vivo*) within siliques of *ger5-2* mutants. Second, our transcriptional analysis of aborting seeds revealed numerous differentially expressed genes associated with carbohydrate metabolism, catabolic processes and autophagy. Third, transcriptional profiling studies indicate

GRAM genes are often up- or down-regulated in diverse tissues (e.g. suspension cells, seedlings, senescing leaves) following changes in sugar supply or carbohydrate status (Buchanan-Wollaston et al. 2005; Osuna et al. 2007; Breeze et al. 2011). Finally, in yeast (*Pichia pastoris*) and mammalian cells, genes encoding GRAM domain proteins are characterized as both positive and negative regulators of autophagy (Oku et al. 2003; Vergne et al. 2009; Zou et al. 2012). As ABA has emerged as specific regulator of autophagy in plant cells, future efforts may be directed towards identifying whether GRAM domain genes fulfill analogous roles in plant growth, development and stress responses (Liu et al. 2009; Vanhee et al. 2011; Honig et al. 2012). Several AuTophaGy-related (*ATG*) and ABA-response genes (*AtHVA22*) identified in the current study have been implicated in programmed cell death (PCD)-like responses leading to reductions in plant fertility (Ghiglione et al. 2008; Chen et al. 2009).

3.5.3 *GER5* and GRAM domain genes in plants

Previous analysis of GRAM genes in Arabidopsis and rice revealed divergent expression patterns amongst fragmentally duplicated members of these gene families (Jiang et al. 2008). In the current study members of the GRAM gene family displayed both unique and overlapping expression domains within reproductive organs, with *GER5*, *GER1* and *GEM* transcripts detected in all tissues examined. Protein-protein interaction studies have further revealed related members of the GRAM family interact with common targets (Takemiya et al. 2012). Additional studies have shown GRAM genes are regulated in a reciprocal manner during development or in response to environmental stimulus (Jiang et al. 2008; Frank et al. 2009). Taken together, these observations indicate a certain degree of functional redundancy or reciprocal regulation may exist

amongst members of the GRAM family in plants. Eukaryotic proteins harbouring GRAM domains (e.g. myotubularins) also form functional homo- or hetero-dimer complexes (Berger et al. 2003; Lorenzo et al. 2006). In Arabidopsis two myotubularin-like genes (*AtMTM1* and *AtMTM2*) encoding GRAM domain proteins were recently identified, with one member proposed to function in the dehydration response of plants (Ding et al. 2012). An intriguing area for future research surrounds the potential for cellular interactions to occur amongst ABA-responsive GRAM proteins and myotubularin-like proteins.

In this study, we have demonstrated a role for *GER5* during the reproductive development of plants and in modifying ABA responses during seed germination. Gene expression, microarray, RNA *in situ* and ABA localization studies have also raised intriguing questions regarding the functional relationship amongst members of the GRAM gene family, as well as the physiological relevance of ABA accumulation in reproductive organs during development and in response to environmental stress.

Table 3.1S Oligonucleotide sequences utilized for genotyping, cloning and gene expression analysis of *GER5*, *GER1* and *GEM*.

Oligonucleotide sequences utilized for genotyping *ger5-2* mutants.

GENE	AGI	Allele	T-DNA Line	Forward Primer (Gene Specific)
GER5	At5g13200	*ger5-2*	GABI_783H10	5'-ACAGAGCACCAACCAGAACC-3'
				Reverse Primer (Gene Specific)
				5'-CCACAAAACCAAACACCAAA-3'
				T-DNA Primer (8409)
				5'-ATATTGACCATCATACTCATTGC-3'

Oligonucleotide sequences utilized for cloning, generation of transgenic lines and RNA *in situ* hybridization experiments.

GENE	AGI		Oligo Sequence
GER5	At5g13200	Forward	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGACAGGATCACAAGAAGACCAA-3'
	(pDONR)	Reverse	5'-GGGGACCACTTTGTACAAGAAAGCTGGGCGTTTTAACCAGACACAGAGCCGTGT-3'
GER5	At5g13200	Forward	5'-GCCATGACAGGATCACAAGAAG-3'
	(pGEM T-Easy)	Reverse	5'-GTTTTAACCAGACACAGAGCCG-3'
GER1	At1g28200	Forward	5'-ATCATGAGTGGGCAAGAGAAT-3'
	(pGEM T-Easy)	Reverse	5'-CCTTCACGGACCATGTGACTGC-3'
GEM	At2g22475	Forward	5'-TTTATGGAGCCGCCGAAGGG-3'
	(pGEM T-Easy)	Reverse	5'-GGAGATCAACATGCTTTTATGG-3'

Table 3.1S (continued) Oligonucleotide sequences utilized for genotyping, cloning and gene expression analysis of *GER5*, *GER1* and *GEM.*

Oligonucleotide sequences utilized PCR and RT-qPCR analysis of mutant and transgenic lines

GEM	At2g22475	Forward	5’-TTGCTCGTTCTCCAGCTGAGACTT - 3’
		Reverse	5'-AATTCTTCCCATTGCAGCATCCGC - 3’
GER1	At1g28200	Forward	5’-CCAGCGGAATCCAATCCTTACGTT - 3’
		Reverse	5’-CCCTGAGCAATTCGTGAAACAGCA - 3’
GER5	At5g13200	Forward	5’-ATACCTGGAGTCGCAAGGCAGAAA - 3’
(FL2)		Reverse	5’-AGTCTCTTTCACCACCACCGGATT - 3’
GER5	At5g13200	Forward	5’-ACAGAGCACCAACCAGAACC-3’
(FL1)		Reverse	5'- TGTAAGAACTCTCTAGACTTAGGA-3'
GER5	At5g13200	Forward	5’-ACAGAGCACCAACCAGAACC-3’
(E1)		Reverse	5’-TTGTTGGATGTTCGACAGGA-3’
GER5	At5g13200	Forward	5’-AATCCGGTGGTGGTGAAAGAGACT-3’
(NTV1)		Reverse	5’-TGTAAGAACTCTCTAGACTTAGGA-3’
PDF subunit	At1g13320	Forward	5'-TAACGTGGCCAAAATGATGC-3'
PP2A		Reverse	5'-GTTCTCCACAACCGCTTGGT-3'
PDF subunit	At1g13320	Forward	5’-CATGTTCCAAACTCTTACCTGCGG-3’
PP2A		Reverse	5’-TGGGTCTTCACTTAGCTCCACCAA-3’
Actin	At3g18780	Forward	5’-TCCCTCAGCACATTCCAGCAGAT-3’
		Reverse	5’-AACGATTCCTGGACCTGCCTCATC-3’

Table 3.2S AGI codes, proposed nomenclature, description and references associated with GRAM domain and myotubularin-related (MTM) genes in *Arabidopsis thaliana*.

AGI code, proposed nomenclature, and description of GRAM domain genes in *Arabidopsis thaliana*

Gene #	AGI	NAME	ALTERNATIVE NAME	Description	Reference
1	At1g02120	VAD1	VAD1 (VASCULAR ASSOCIATED DEATH1)	pathogen response, cell death, ethylene and SA signaling	Lorrain et al. 2004 Bouchez et al. 2007
2	At1g03370	unnamed		contains C2 domain	
3	At1g28200	GEM-Related 1 (GER1)	FIP1 (FH INTERACTING PROTEIN1)	interacts with AFH1, involved in cytoskelelal dynamics	Banno and Chua 2000
4	At2g22475	GEM	GLABRA2 EXPRESSION MODULATOR	epidermal patterning, cell cycle, chromatin modification	Caro et al. 2007
5	At3g59660	ROR1	ROR1 (ROSCOVITINE RESISTANT1)	cytokinin metabolism, contains C2 domain	Dwivedi et al. 2010
6	At4g01600	GEm-Related 2 (GER2)			
7	At4g40100	GEm-Related 3 (GER3)	PRLS1 (PP1 regulatory subunit 2-like protein 1)	blue light signaling in stomata	Takemiya et al. 2012
8	At5g08350	GEm-Related 4 (GER4)			
9	At5g13200	GEm-Related 5 (GER5)	ABR1 (ABA-RESPONSIVE 1)	pathogen response, cell death, ABA/SA signaling reproductive development	Choi and Hwang 2011
10	At5g23350	GEm-Related 6 (GER6)			
11	At5g23360	GEm-Related 7 (GER7)			
12	At5g23370	GEm-Related 8 (GER8)			
13	At5g50170	unnamed		contains C2 domain	

AGI code, nomenclature, and description of myotubularin-related genes encoding GRAM domain proteins

1	At3g10550	AtMTM1	ARABIDOPSIS MYOTUBULARIN1	dehydration and drought response, PtdIns*P* metabolism chromatin modification	Ding et al. 2009; 2012
2	At5g04540	AtMTM2	ARABIDOPSIS MYOTUBULARIN2	dehydration and drought response, PtdIns*P* metabolism	Ding et al. 2012

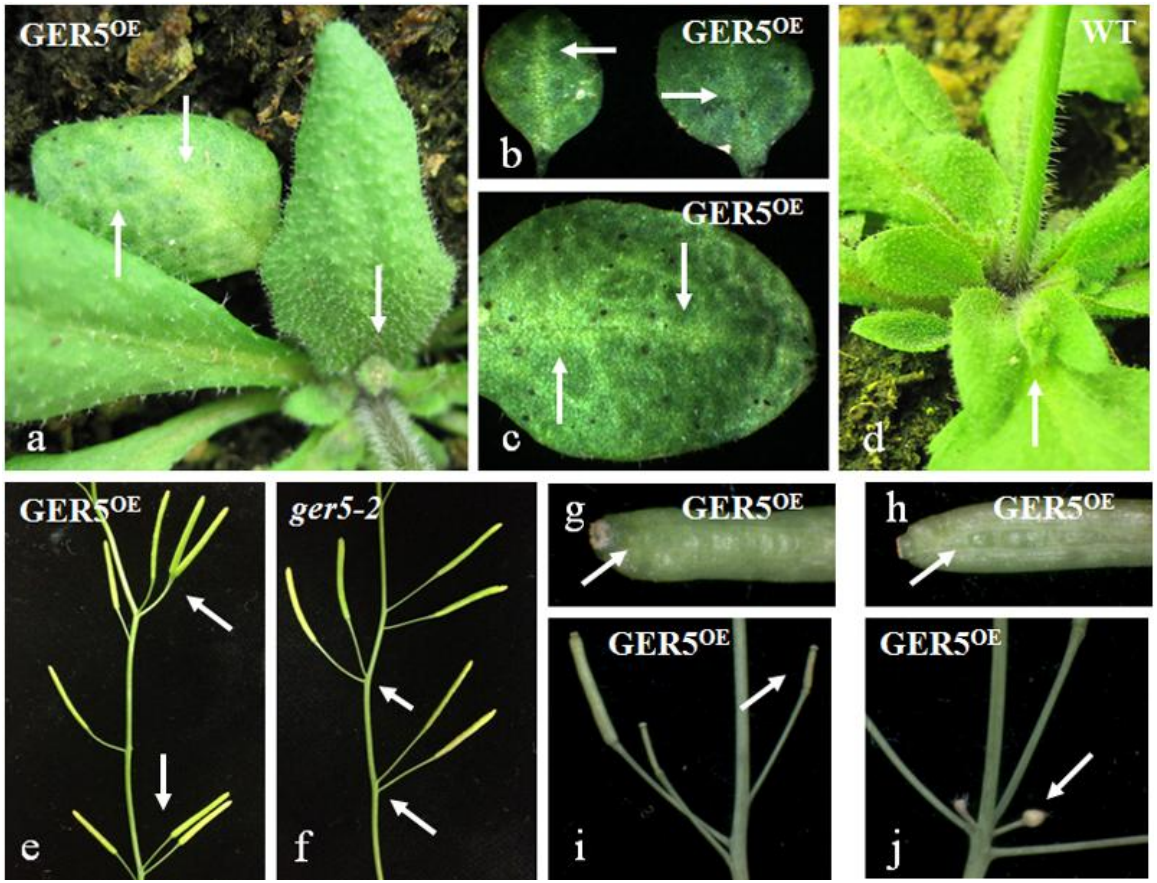

Fig. 3.1S Reproductive phenotypes observed in mutant and transgenic lines. Chlorosis and axillary meristems of *GER5*^{OE} lines. **a** Veinal chlorosis of mature rosette leaves and abnormal axillary meristems (see arrows). Axillary meristems gives rise to aborted flowers shown in Fig. 3.2. **b** and **c** Veinal chlorosis upon mature rosette leaves following emergence of the primary inflorescence meristem (see arrows). **d** Healthy axillary meristem emerging from wild-type plant. **e** and **f** Silique clusters and phyllotaxy defects in *GER5*^{OE} lines and *ger5-2* mutant. (**g** and **h**) *GER5*^{OE} lines develop extra carpels under normal growth conditions. **i** and **j** Aborted flowers and unfertilized siliques emerge from inflorescence stalk of *GER5*^{OE} lines.

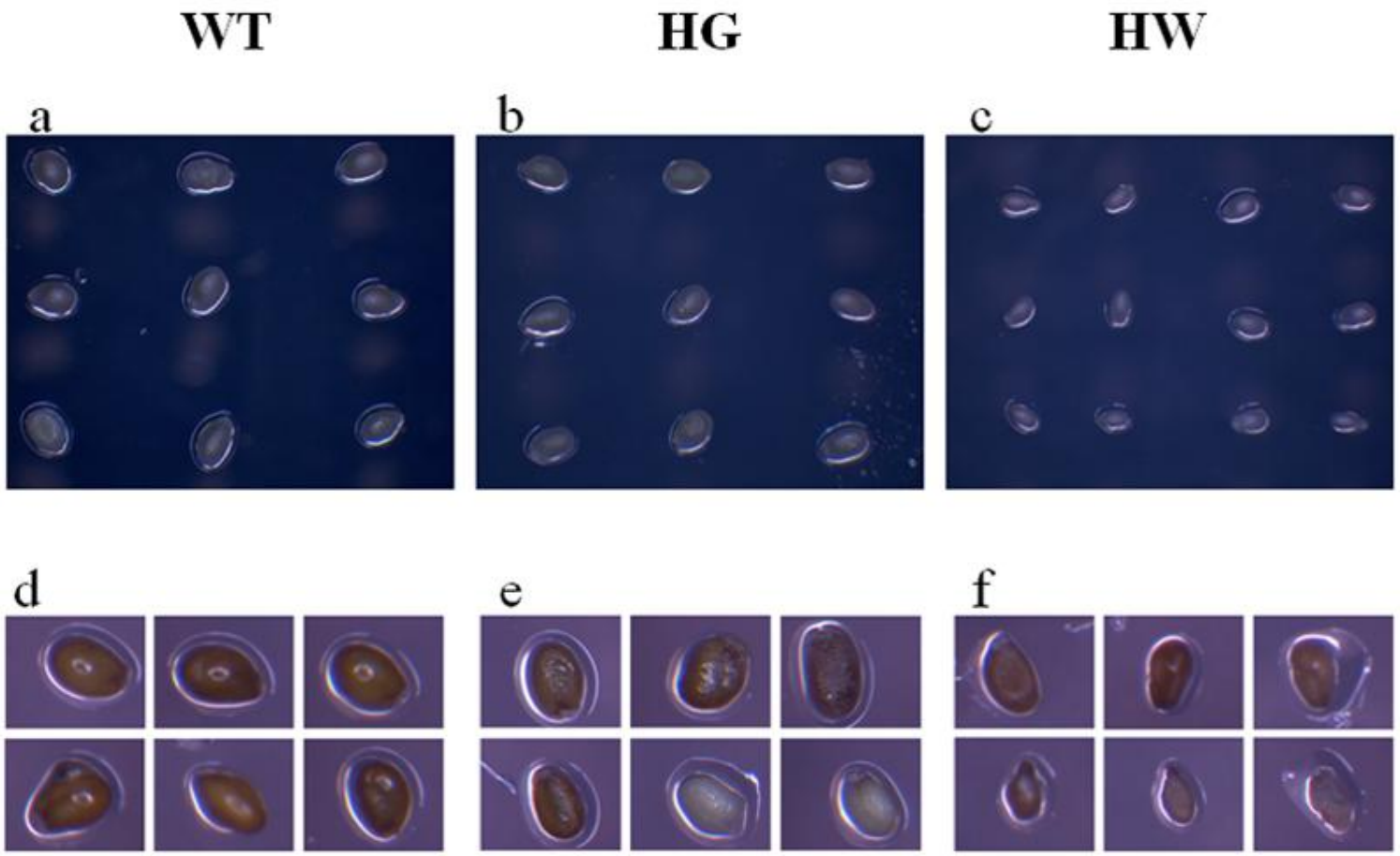

Fig. 3.2S *In vitro* ovule culture technique utilized to obtain viable seedlings from *ger5-2* mutants (HG and HW) (See Fig 3.2b,e). **a-c** Healthy or abnormal seeds isolated from *ger5-2* mutant or wild-type plants were placed on media (10% sucrose, 0.5x MS salts, 0.05% MES, 0.3% phytagel, 400 mg l^{-1} glutamine pH=5.9) for 5d upside down in the darkness. **d-f** Seed categories were then transferred to Arabidopsis medium (AM) (Sauer and Friml 2004), stratified at 4°C for 2d, and assessed for germination and seedling growth for 2-4 weeks. After culture a large proportion HW seeds were shrunken and did not contain viable embryos.

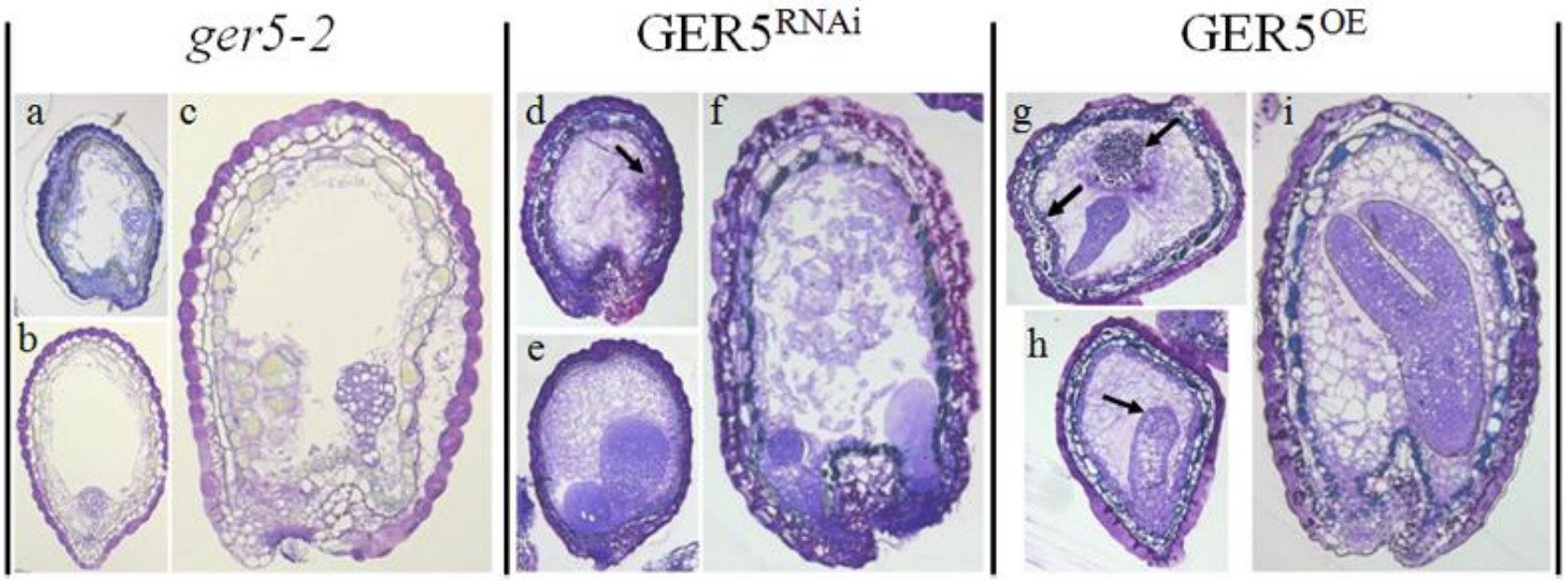

Fig. 3.3S Severe seed development defects observed in ovules extracted from *ger5-2* mutant and transgenic lines ($GER5^{RNAi}$, $GER5^{OE}$). **a-c** HW seeds isolated from *ger5-2* mutants frequently arrest at the globular stage of embryo development. **d-f** Abnormal seeds isolated from siliques of $GER5^{RNAi}$ lines. **d** Seed lacking embryo with cellularized endosperm. Solid arrow identifies starch granules which have accumulated in endosperm. **e** Proliferation of embryo and chalazal endosperm. Enlarged embryo undergoes multiple cell divisions, but lacks differentiation of cell layers internal to the protoderm. **f** Embryo arrest coincides with proliferation of chalazal endosperm. Starch granules accumulate in cell layers corresponding to the outer integument. **g-h** Seed development defects observed in $GER5^{OE}$ lines. **g** Arrest of globular stage embryo. Solid arrows identify starch granules which accumulate within endosperm and aleurone layer. **h** Arrested embryo with intact protoderm. Cell layers corresponding to ground meristem and procambium are disorganized. Solid arrow identifies protoderm **i** Starch granules accumulate in cell layers corresponding to outer integuments. Embryo arrested at torpedo stage of development.

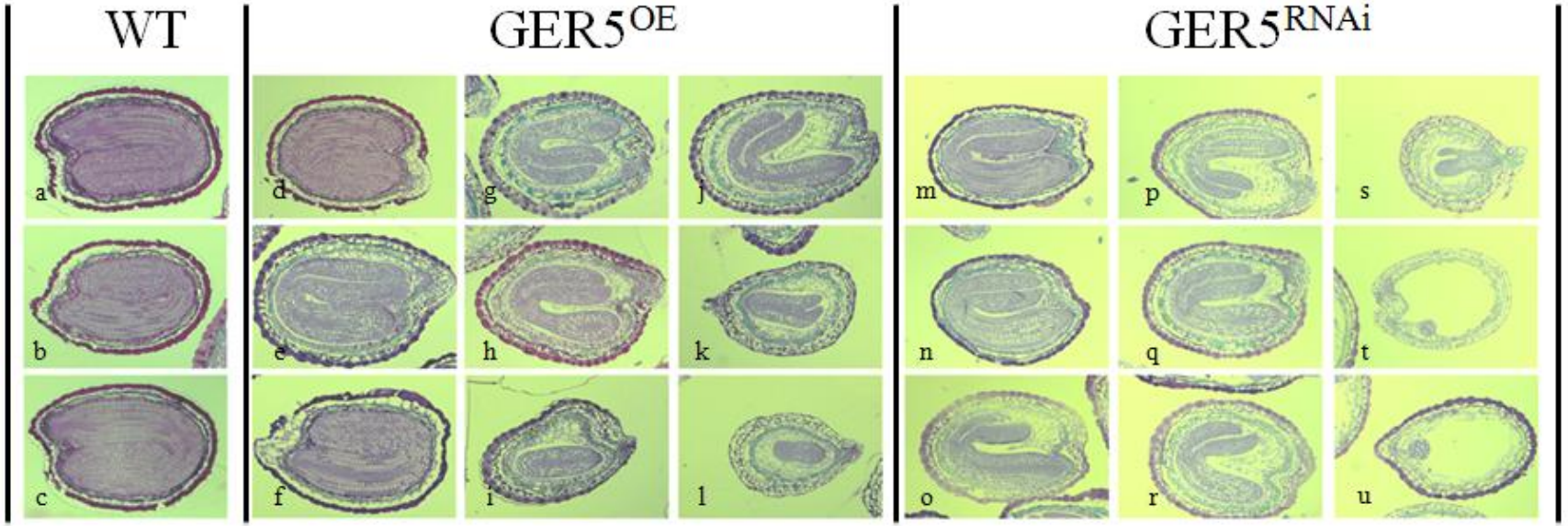

Fig. 3.4S Range of seed development defects in transgenic lines ($GER5^{OE}$, $GER5^{RNAi}$). **a-c** Wild-type seeds. **d-l** Left-to right, seeds isolated from $GER5^{OE}$ lines display a range of phenotypes ranging from mild to severe. Extracted seeds contain embryos which arrested at torpedo and walking-stick stages of embryo development. **m-u** Left-to-right, range of seed development and embryo defects observed in $GER5^{RNAi}$ lines. Similar to *ger5-2* mutants, embryos isolated from $GER5^{RNAi}$ lines arrest at earlier stages (globular) of embryo development. **s-u** Note differences in differentiation of outer integuments.

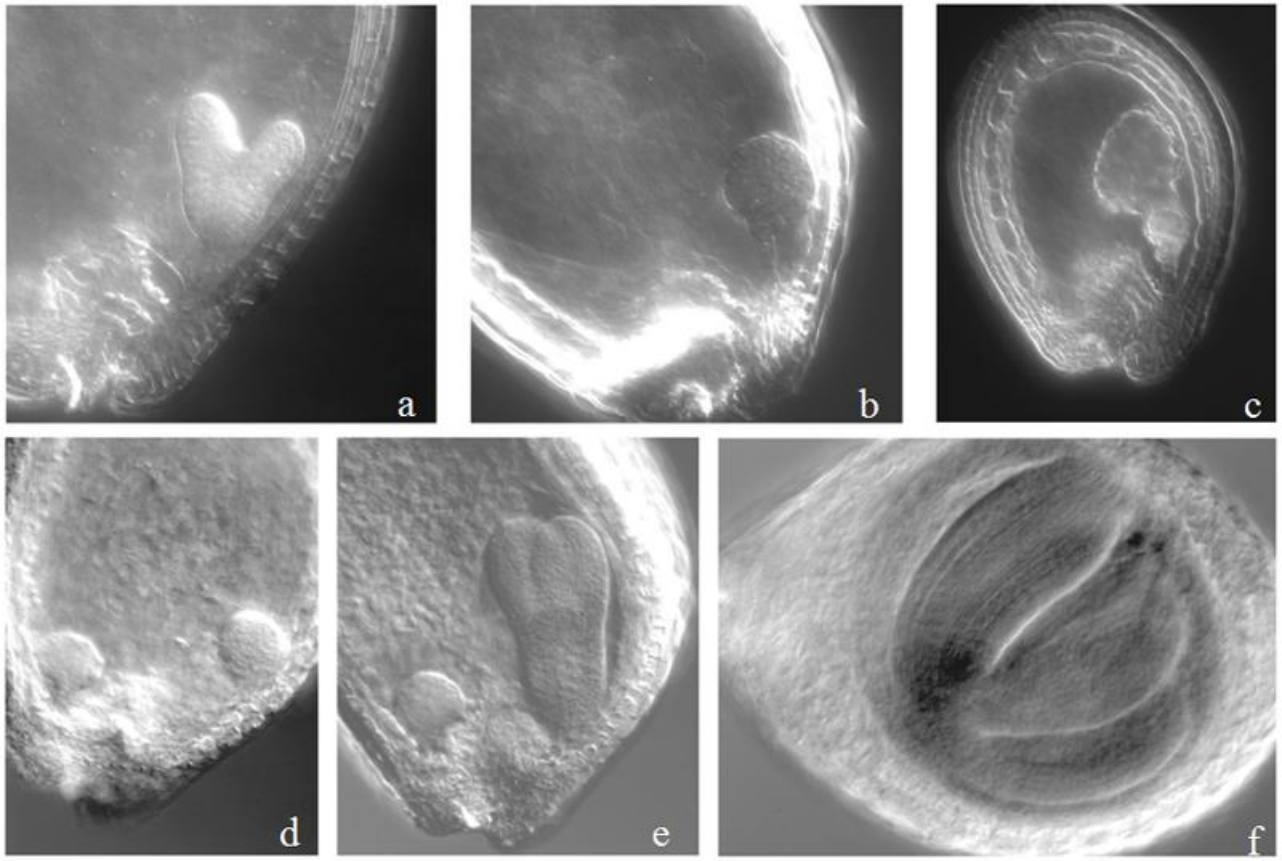

Fig. 3.5S Microscopic analysis of WT, *ger5-2* mutant and transgenic ($GER5^{OE}$, $GER5^{RNAi}$) seeds. **a,b** WT and *ger5-2* mutant (HW) seeds isolated from comparable siliques 5 days after pollination. **a** WT seeds have progressed to heart stage of embryo development. **b** Abnormal seeds extracted and cleared from *ger5-2* mutants arrest at globular stage of embryo development. **c** Whole-mount sample of *ger5-2* seed (HW) arrested at globular stage of development. **d** and **e** Embryo arrest at globular or heart stage of development in $GER5^{RNAi}$ lines is accompanied by proliferation of chalazal endosperm. **f** Abnormal, arrested seed isolated from $GER5^{OE}$ line.

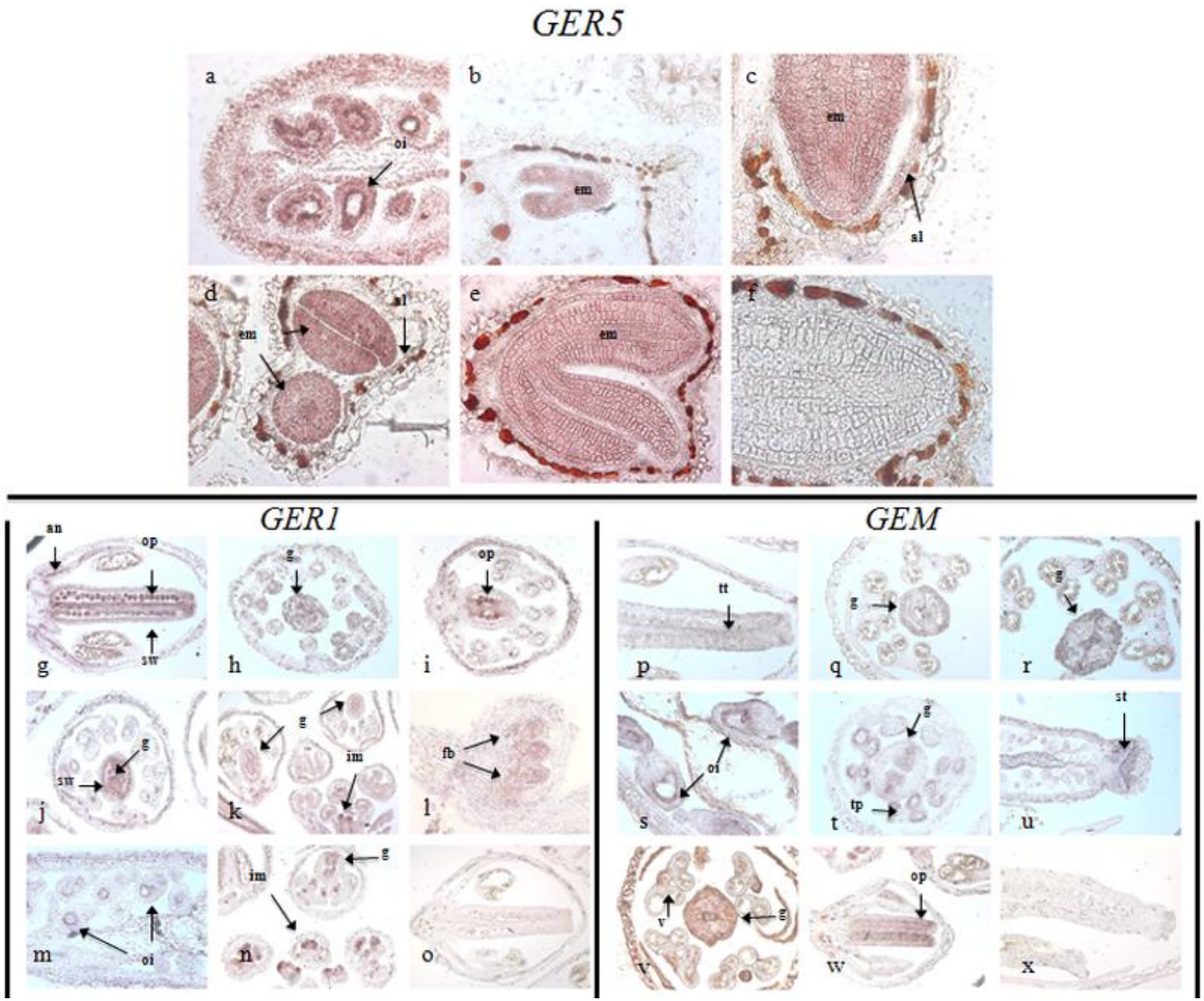

Fig. 3.6S *GER5*, *GER1* and *GEM* transcript localization in reproductive organs of *Arabidopsis thaliana*.

Fig. 3.6S (continued) a *GER5* expression in ovule integuments prior to fertilization. **b-e** *GER5* expression detected throughout heart and bent cotyledon stage embryos in seeds. *GER5*-specific signals detected in aleurone tissues. **f** Negative control hybridization with *GER5* sense probe. **g-n** *GER1* expression in flowers and reproductive organs of Arabidopsis. **g** *GER1* transcripts localize to ovule primordia, silique wall and vascular tissue of anthers. **h** *GER1* localizes to gynoecium and **i** ovule primordia. **j** *GER1* localization throughout gynoecium and silique wall. **k** *GER1* transcripts detected in inflorescence meristem, gynoecium and **l** floral buds. **m** *GER1*-specific signals associate with ovule integuments, in addition to **n** inflorescence meristem and gynoecium. **o** Negative control hybridization with *GER1* sense probe. **p-w** *GEM* expression in flowers and reproductive organs of Arabidopsis. **p** *GEM*-specific transcripts detected within transmitting tract. **q** and **r** *GEM* expression throughout gynoecium. **s** *GEM* expression in ovule integuments. **t** *GEM* expression in gynoecium and tapetum. **u** *GEM* transcripts localize to stigma. **v** *GEM*-specific signals detected in vascular tissues of anthers and gynoecium. **w** *GEM* transcripts localize to ovule primordia. **x** Negative control hybridization with *GEM* sense probe. oi ovule integument, em embryo, al aleurone, an anther, op ovule primordial, sw silique wall, g gynoecium, fb floral bud, im inflorescence meristem, tt transmitting tract, st stigma, tp tapetum.

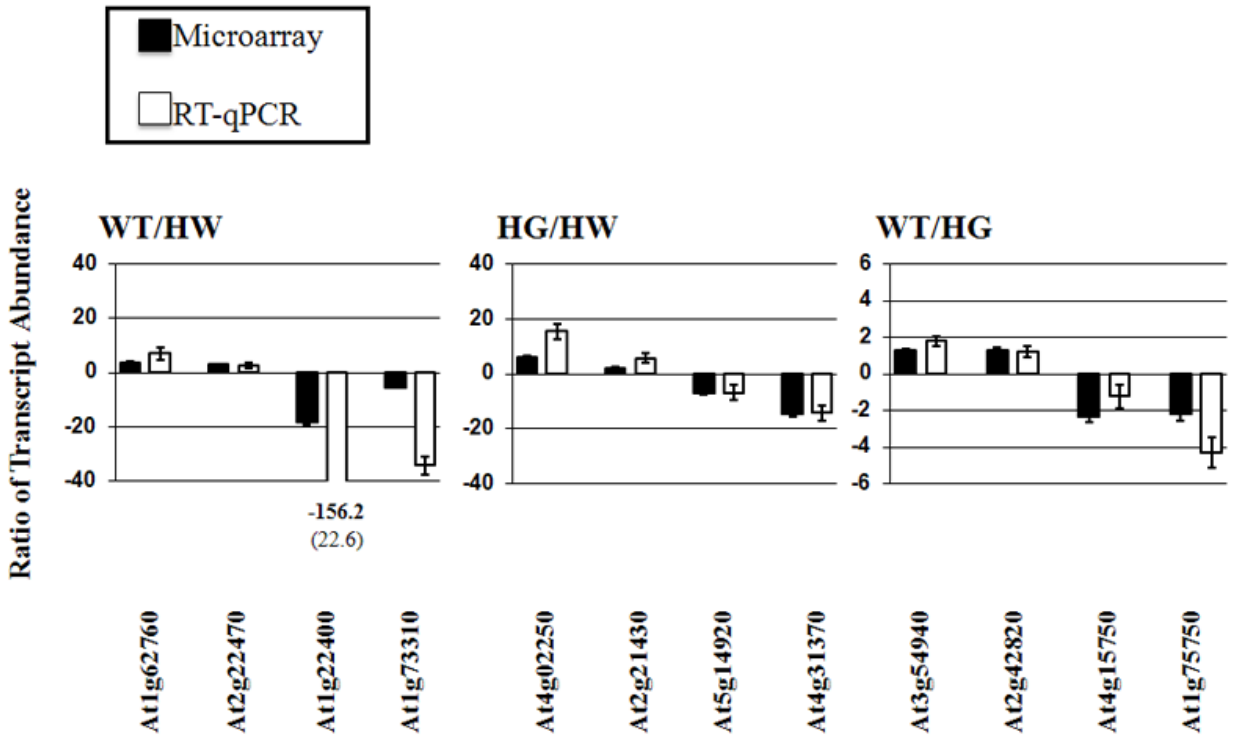

Fig. 3.7S RT-qPCR validation of microarray results. For direct comparisons between seed categories select genes were measured by RT-qPCR and normalized to the expression of the internal reference gene *PP2A* (At1g13320). RT-qPCR data represent the fold change (± SD) from all four biological replicates. Microarray data represent fold change (± SE) of 4 (or fewer) biological replicates. In general, microarray and RT-qPCR were correlated. However, for direct comparisons of WT/HG versus HW seeds, fold change values obtained by RT-qPCR were consistently equal to or greater than fold change values obtained by microarray. Differences in fold change obtained between microarray and RT-qPCR may be attributed to the reduced accuracy of microarray in quantifying low-abundance transcripts or the reduced dynamic range of microarray relative to RT-qPCR.

4.0 Hormonal and environmental regulation of AuTophaGy-related (*ATG*) and ABA-response genes in autotrophic and heterotrophic plant organs

4.1 Abstract

Autophagy is an important catabolic process responsible for degrading cytoplasmic components and recycling nutrients during starvation. Recent studies indicate abscisic acid (ABA) functions as a specific regulator of autophagy in plants. However, there remains considerable uncertainty regarding the contribution of ABA-dependent or ABA-independent signaling pathways towards activating autophagy in response to environmental stress. ABA and sugar signaling pathways also converge in plants, and there is evidence that shared components of these pathways are central regulators of anabolic (e.g. ribosome biogenesis, translation) and catabolic processes (e.g. protein degradation, autophagy) during plant growth and development. In the current study, we have assessed the transcriptional response of characterized positive and negative regulators of autophagy in both vegetative (source) and reproductive (sink) tissues of *Arabidopsis thaliana* exposed to cold and heat stress. In addition, *A*u*T*opha*G*y-related (*ATG*) genes were assessed in mutant backgrounds deficient in ABA biosynthesis and sugar signaling. In response to abiotic stress, and in particular cold stress, *ATG* genes were activated in both vegetative and reproductive organs, which coincided with an up-regulation of transcription factors involved in ABA-independent and sugar (ABA) signaling pathways. However, in mutant backgrounds deficient in ABA biosynthesis, these same *ATG* and ABA response genes were often unaffected or displayed tissue-specific expression patterns which contrasted with their response to environmental stress.

At a morphological level, monodanyslcadaverine (MDC) staining to label autophagosomes or autophagic bodies identified cell types where autophagy was highly active. Together, these results suggest temperature stress alters the autophagic response in both autotrophic and heterotrophic plant organs, and provides a framework for future studies evaluating the physiological function of autophagy in plant development and stress responses.

4.2 Introduction

In yeast, animal and plant cells, autophagy, meaning 'self-eating', is a major catabolic bulk degradation system utilized to recycle cellular components such as proteins, organelles and cytosolic macromolecules (Liu and Bassham 2012). Relative to the ubiquitin (Ub)/26S proteasome system (UPS), which selectively targets short-lived regulatory proteins for degradation, autophagy is characterized by sequestration of the cytoplasm by endomembranes to form an autophagic vacuole or autophagosome. In turn, cellular components engulfed within autophagic vacuoles or autophagsomes are delivered in bulk to the lysosome/vacuole for degradation by vacuolar hydrolases and proteases (Li and Vierstra 2012; Liu and Bassham 2012). Our understanding of autophagy in plants is strongly influenced by our knowledge of autophagic pathways in yeast (*Saccharomyces cerivisae*, *Pichia pastoris*) and the identification and characterization of orthologous components (e.g. *ATG*, *TOR*) in mammals and plants (Bassham 2009). In plants autophagy is frequently associated with the response of cells to nutrient starvation (carbon, nitrogen), natural senescence, or pathogen infection (Xiong et al. 2005; Lenz et al. 2011; Guiboileau et al. 2012). Beyond carbon and nitrogen stress, plant autophagy

also functions to degrade oxidized proteins and plays an important role in mediating plant tolerance to environmental stress (Xiong et al. 2007; Liu et al. 2009).

Although core components of the autophagy machinery are conserved across eukaryotes, current research in the plant autophagy field indicates phylum-specific components also exist (Izumi et al. 2010; Honig et al. 2012). Several reports have identified abscisic acid (ABA) as a key regulator of autophagy during plant development and stress responses (Chen et al. 2009; VanHee et al. 2011; Honig et al. 2012). However, there is still much uncertainty regarding the role of ABA-dependent or ABA-independent signaling pathways in activating or repressing autophagic pathways in plants (Bassham 2009; Liu et al. 2009).

During the course of reverse genetic studies seeking to functionally characterize ABA and stress-responsive members of the GRAM gene family in *Arabidopsis thaliana*, we identified numerous transcriptional profiling studies where GRAM genes were coordinately expressed during natural senescence or in response to carbon starvation (Buchanan-Wollaston et al. 2005; van der Graaf et al. 2006; Usadel et al. 2008; Breeze et al. 2011). In these same studies, changes in GRAM gene expression were frequently accompanied by changes in ABA metabolism and signaling, in addition to upregulation of core *ATG* genes. In yeast and humans, several genes encoding GRAM domain proteins (e.g. UGT51, MTMR9, MTMR14) are characterized as positive or negative regulators of autophagy (Oku et al. 2003; Vergne et al. 2009; Zou et al. 2012). Based on the above observations, we reasoned members of the ABA-responsive GRAM gene family may also function as positive or negative regulators of autophagy in plants. Several characterized negative regulators of autophagy (*TOR*, *AtHVA22*) are also

responsive to ABA and are preferentially expressed within rapidly growing embryonic and meristematic tissues of plants (Chen et al. 2002; Menand et al. 2002; Deprost et al. 2007; Chen et al. 2009). In addition, genetic and physiological studies have demonstrated autophagy facilitates recycling and remobilization of nutrients during the reproductive stage of plant development (Deprost et al. 2007; Ghiglione et al. 2008; Guiboileau et al. 2012). However, our understanding of autophagy at this stage of the plant lifecycle remains incomplete.

To date the majority of transcriptional or morphological studies of autophagy in plants have focused upon the response of cultured cells or protoplasts to carbohydrate deprivation (Chen et al. 1994; Contento et al. 2004; 2005; Rose et al. 2006). To gain further insight into the role of ABA as a mediator of autophagy at the reproductive stage of development, we have analyzed the transcriptional response of *ATG* genes in both source (autotrophic) and sink (heterotrophic) organs exposed to environmental stress, and also evaluated these same genes within mutant backgrounds defective in ABA biosynthesis and sugar signaling. In addition, using the autophagosome-specific dye monodansylcadaverine (MDC), we have identified specific cell types where autophagic pathways are active during plant development. Taken together, our results provide new information regarding the contribution of ABA biosynthesis and signaling towards the regulation of autophagy during plant development and in response to environmental stress.

4.3 Materials and methods

4.3.1 Plant material and growth conditions

Wild-type (WT) and hormone biosynthesis and signaling mutants (*aba1-5*, *aba2-1*, and *ctr1-1*, all in Col-0 background) used for gene expression studies and detection of autophagosomes were obtained from the Arabidopsis Biological Resource Centre (ABRC). For gene expression studies wild-type and mutant plants were grown on soil under long day conditions (16 h light) at 22℃ and relative humidity of 70%. For gene expression studies, imposition of cold (0.0 ± 2.0°C) and heat stress (37.0 ± 2.0°C) treatments and sampling of plant material from reproductive stage plants was conducted according to Baron et al. (2012). For detection of autophagosomes in roots, 7-day old seedlings germinated on half-strength MS medium (1% sucrose) and grown under long day conditions would be transferred to identical MS media lacking sucrose, and subsequently transferred to the dark for 48 h. For autophagosome detection in epidermal peels and developing seeds, cauline leaves (non-senescing) or siliques (10 days after pollination) were removed from reproductive stage plants corresponding to growth stages 6.3-6.5 (Boyes et al. 2001), placed on moist filter paper in petri dishes and incubated in the dark for 48 h before exposure to cold (0.0℃) or heat (37.0℃) stress for 6-8 h.

4.3.2 RNA isolation and RT-qPCR analysis

Total RNA was extracted from cauline leaves and inflorescence meristems using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) with genomic DNA removed through on-column digestion using an RNase-free DNAase Kit (Qiagen) according the manufacturer's protocol. An aliquot of total RNA was subsequently analyzed on a Nano-drop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Depending on the tissue 1-5 μg of total RNA was utilized to synthesize first-strand cDNA using a Maxima™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada) for RT-qPCR. Following synthesis cDNA concentration was determined and cDNA diluted to an appropriate volume. Quantitative RT-PCR was carried out using the Sso Fast™ Evagreen® SuperMix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) with a final reaction volume of 20 μL (10 μL master mix, 1 μL cDNA, 9 μL primer mix). In a single experiment, each sample was assayed in triplicate and expression levels were calculated relative to the internal reference gene phosphatase 2A subunit (*PP2A* = At1g13320) (Czechowski et al. 2005). Triplicate PCRs (technical) were conducted on two or three separate cDNA samples (biological) with each cDNA representing RNA extracted from 3-6 plants. During data analysis wells producing abnormal amplification plots or dual melting curves would be eliminated from subsequent analyses. RT-qPCR data was analyzed according to the comparative Ct ($^{2-\Delta\Delta Ct}$) method, where ΔC_T is calculated for each sample as the difference between the *Ct* of a gene of interest and the *Ct* of a reference gene (e.g. *PP2A*). Oligonucleotide sequences utilized for RT-qPCR experiments are presented in Table 4.1S.

4.3.3 MDC staining and microscopy

Protocols for staining and visualizing autophagosomes with monodansylcadavarine (MDC) were adapted from Contento et al. (2005) with modifications described below. MDC-stained autophagosomes were visualized *in vivo* using a Leica DMR microscope (Leica Microsystems Inc., Concord, ON, Canada) equipped with a Leica DC500 digital camera for both light and UV-fluorescence microscopy. MDC-stained epidermal peels, developing seed coats or aleurone cells were

visualized using a DAPI-specific filter. For epidermal peels, cauline leaves (fully expanded, no visible signs of senescence) were isolated from reproductive stage plants (growth stage 6.3-6.5), placed on petri-plates, and incubated in darkness for 48 h to artificially induce senescence. Epidermal peels were then incubated with 50 μM MDC for 15 min at room temperature, washed twice with phosphate-buffered saline (PBS) to remove excess dye, and analyzed by fluorescence microscopy. For MDC staining of seed coats, siliques (10 days after pollination) were isolated from reproductive stage plants described above. For visualizing autophagosomes in response to abiotic stress, seedlings, cauline leaves or siliques were first incubated in darkness for 48 h, followed by exposure to temperature treatments for 6-8 h.

4.4 Results

4.4.1 Expression of AuTophaGy-related (*ATG*) genes

In the current work, we have assessed the transcriptional regulation of *ATG* and ABA-response genes in both source and sink organs of Arabidopsis exposed to temperature stress, in addition to mutant backgrounds deficient in ABA biosynthesis or sugar signaling (*aba1*, *aba2*, *ctr1*). Four *ATG* genes were chosen for assessment in the current study, all of which have been functionally characterized in Arabidopsis. *ATG8e* encodes an autophagosome-membrane protein which conjugates to phosphatidylethanolamine (PE) and is frequently used as an autophagosome marker (Contento et al. 2005). *ATG7* encodes an ATP-dependent E1 enzyme responsible for activation of ATG8/ATG12 prior to conjugation (Doelling et al. 2002). *ATG18a* is essential for autophagosome formation in response to natural senescence, nutrient stress and abiotic stress (Xiong et al. 2005; Liu et al. 2009). *AtTOR* is a highly conserved

phosphatidylinositol 3-kinase related kinase which perceives nutrient availability and negatively regulates autophagy under nutrient replete conditions (Liu and Bassham 2010).

In cauline leaves of plants exposed to cold stress, transcripts of *ATG7*, *ATG8e* and *ATG18a* increased 1.8-fold to 2.2-fold (Fig. 4.1a,c,e). However, within this same tissue, steady-state mRNA levels of *ATG7*, *ATG8e* and *ATG18a* were unaffected by heat stress (Fig. 4.1a,c,e). In inflorescence meristems *ATG8e* and *ATG18a* transcripts increased in response to both cold and heat stress (Fig. 4.1d,f), whereas *ATG7* levels were unaffected by either temperature extreme (Fig. 4.1b). In response to cold and heat stress, upregulation of *ATG8e* and *ATG18a* within inflorescence meristems also coincided with repression of *AtTOR*, an upstream negative regulator of autophagy (Fig. 4.1d,f,h). In cauline leaves exposed to heat stress, where *ATG7*, *ATG8e* and *ATG18a* transcripts levels were unaffected, *AtTOR* level was slightly elevated (Fig. 4.1a,c,e,g).

In contrast to the accumulation of *ATG8e* and *ATG18a* transcripts within inflorescence meristems exposed to cold and heat stress, no significant differences in *ATG7*, *ATG8e* or *ATG18a* expression were observed within inflorescence meristems of ABA-deficient mutants (Fig. 4.1b,d,f). However, within cauline leaves of these same mutants several *ATG* genes were up- or down-regulated depending upon the mutant and gene assessed (Fig. 4.1 a,c,e). For example, where *ATG7*, *ATG8e* and *ATG18a* were upregulated in cauline leaves exposed to cold stress, in ABA-deficient backgrounds *ATG7* expression increased, whereas *ATG18a* was repressed (Fig. 4.1a,e). *AtTOR* expression was also elevated in cauline leaves of both ABA-deficient mutants in addition to the *ctr1* mutants (Fig. 4.1g).

4.4.2. Expression of ABA and stress-responsive *AtHVA22* genes

In barley (*Hordeum vulgare*) and Arabidopsis, members of the *HVA22/AtHVA22* gene families are responsive to ABA and abiotic stress and have been functionally characterized as negative regulators of programmed cell death or autophagy (Guo and Ho 2008; Chen et al.2009). In the current study, with the exception of *AtHVA22a* expression in inflorescence meristems, cold stress triggered a strong and coordinated accumulation of *AtHVA22a*, *AtHVA22d*, *AtHVA22e* transcripts in both source and sink organs (Fig. 4.2a-f). However, the fold-induction of *AtHVA22d* and *AtHVA22e* genes (12.1- to 25.3-fold) greatly exceeded the induction of *ATG7*, *ATG8e* and *ATG18a* by cold stress in these same tissues (Fig. 4.1). In contrast, no member of the *AtHVA22* gene family was up-regulated by heat stress and for select genes (*AtHVA22a*, *AtHVA22e*), reduced transcript levels were observed (Fig. 4.2b,e).

In mutant backgrounds, members of the *AtHVA22* gene family displayed complex tissue-specific patterns of expression that contrasted with the coordinated induction of this gene family in response to cold stress (Fig. 4.2a-f). For example, within cauline leaves of *aba2* mutants *AtHVA22a* and *AtHVA22d* transcripts were elevated (Fig. 4.2a,c), whereas for inflorescence meristems from identical plants, transcripts of these same genes were reduced (Fig. 4.2b,d). *AtHVA22a* and *AtHVA22d* expression was also down-regulated in inflorescence meristems of *aba1* mutants (Fig. 4.2b,d). Similarly, within inflorescence meristems of *ctr1* mutants, *AtHVA22d* transcripts decreased, whereas in cauline leaves this same gene was up-regulated (Fig. 4.2c,d). In comparison *AtHVA22e* was repressed in cauline leaves of *aba1*, *aba2* and *ctr1* mutants but was unaffected within inflorescence meristems isolated from these same plants (Fig. 4.2e,f).

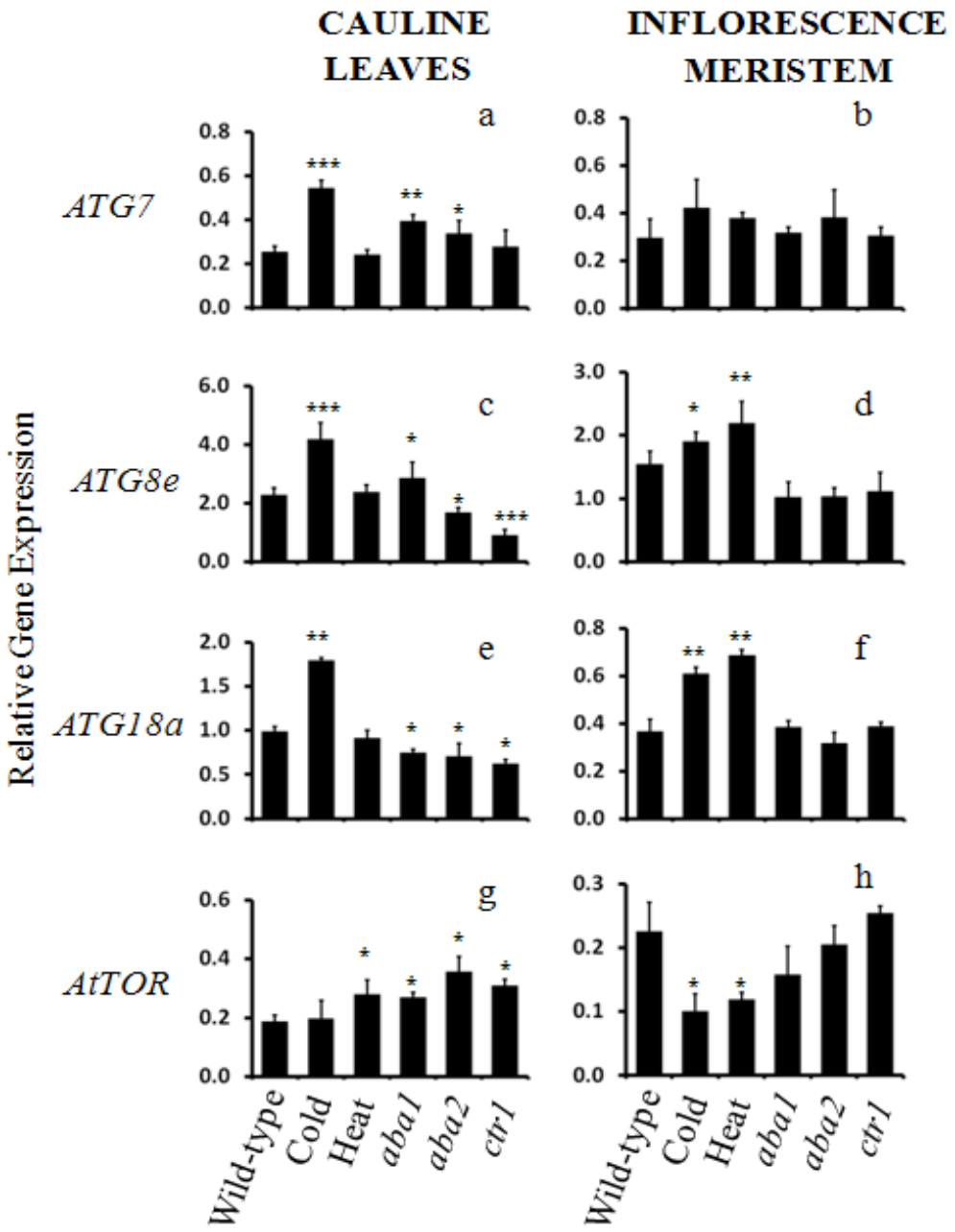

Fig. 4.1 Expression of AuTophaGy-related (*ATG*) genes in Arabidopsis. Figures **a** through **h** display the expression level of genes in cauline leaves and inflorescence meristems of plants at the reproductive stage of development. Wild-type, *aba1*, *aba2* and *ctr1* mutants were grown under identical conditions (22°C). For abiotic stress treatments a subset of wild-type plants were exposed to temperature stress (cold = 0°C, heat = 37°C) for 24 h. For a given gene (*ATG7, ATG8e, ATG18a, AtTOR*) and tissue asterisks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to wild-type plants grown at 22°C. Data correspond to mean and standard deviation (SD) of three biological replicates. RT-qPCR data were normalized to the expression of the internal reference gene *PP2A* (*At1g13320*). P-values determined according to a two-tailed studentized t-test (equal variance).

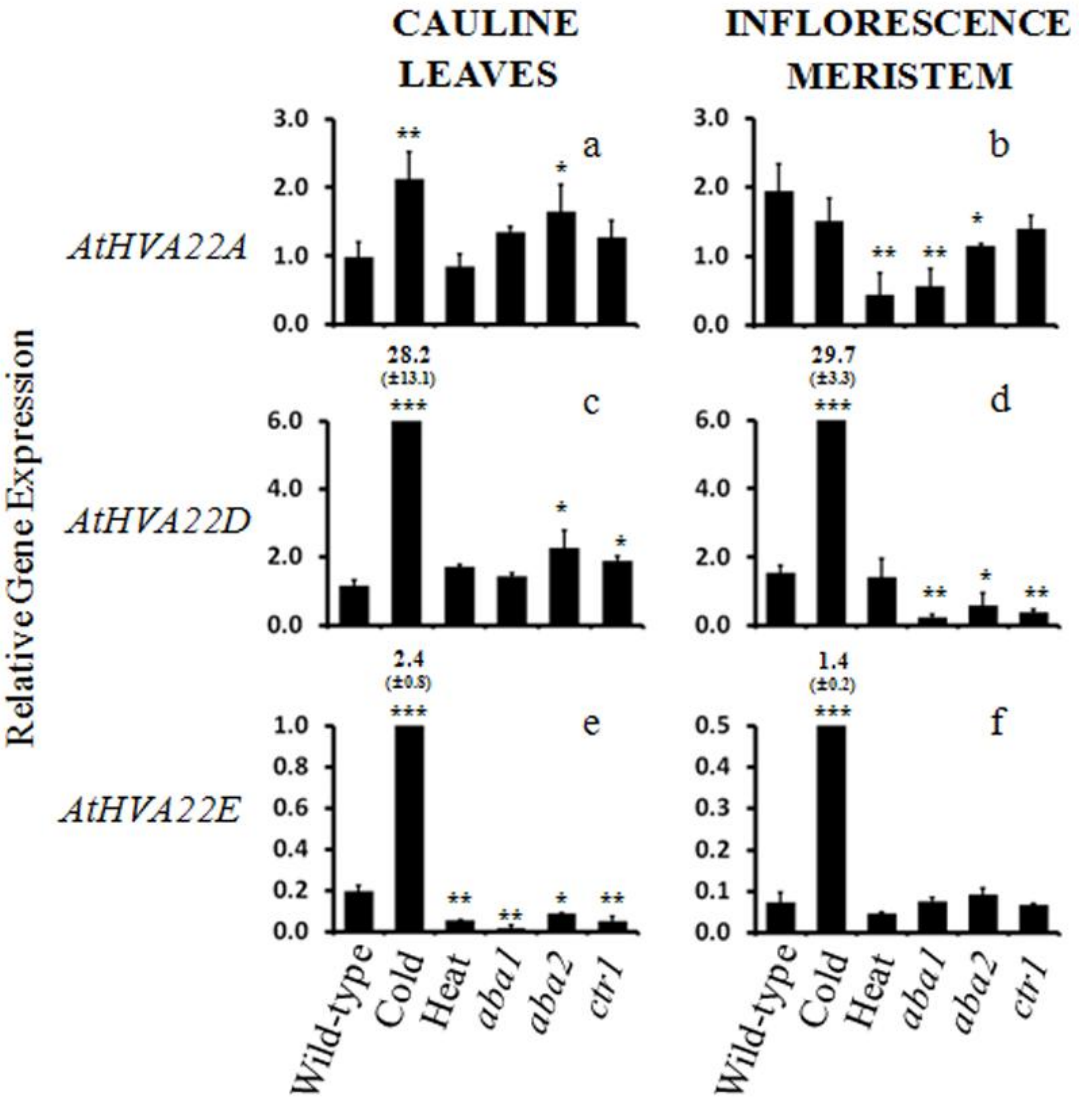

Fig. 4.2 Expression of the *AtHVA22* gene family in Arabidopsis. Figures **a** through **f** represent the expression level of genes in cauline leaves and inflorescence meristems of reproductive stage plants. Wild-type, *aba1*, *aba2* and *ctr1* mutants were grown under identical conditions (22°C). For abiotic stress treatments a subset of wild-type plants were exposed to severe temperature stress (cold = 0°C, heat = 37°C) for 24 h. For a given gene (*AtHVA22a, AtHVA22d, AtHVA22e*) and tissue (cauline leaves, inflorescence meristem) astericks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to wild-type plants grown at 22°C. Data correspond to mean and standard deviation (SD) of three biological replicates. RT-qPCR data were normalized to the expression of the internal reference gene *PP2A* (*At1g13320*). P-values determined according to a two-tailed studentized t-test (equal variance).

4.4.3 Expression of transcription factors associated with ABA-independent signaling pathways

At present considerable uncertainty surrounds the contribution of ABA-dependent or ABA-independent signaling pathways in inducing autophagy across a wide array of biotic or abiotic stress conditions (Xiong et al. 2007; Liu et al. 2009; 2012). In the current study, we sought to examine how transcription factors associated with ABA-independent signaling (abiotic stress) or ABA-mediated sugar signaling pathways were regulated under conditions conducive to induction of autophagy.

In both cauline leaves and inflorescence meristems exposed to cold stress, expression of *C-REPEAT BINDING ELEMENT FACTOR 2* (*CBF2*), a transcriptional activator associated with the cold response pathway, was elevated 132-fold and 300-fold, respectively, over plants grown at 22°C (Fig. 4.3a,b). Similarly, for these same tissues, expression of *CBF1* and *CBF3*, two genes closely related to *CBF2*, were also strongly upregulated (17.5-54.6-fold, $P<0.001$) by cold stress, although not to the same extent as observed for *CBF2* (data not shown). Within inflorescence meristems *CBF2* expression increased slightly in response to heat stress (Fig. 4.3b). However, *CBF1-3* expression was generally unaffected or even slightly repressed by heat stress (Fig. 4.3a, data not shown). In Arabidopsis, *DREB2A* interacts with *cis*-acting dehydration-response elements (DRE) and activates genes involved in drought and salt stress (Sakuma et al. 2006). In response to cold stress, *DREB2A* was up-regulated in cauline leaves (14-fold) and inflorescence meristems (8.0-fold) (Fig. 4.3c,d). In response to heat stress, *DREB2A* expression was unmodified in cauline leaves, but upregulated 3.2-fold in inflorescence meristems (Fig. 4.3c,d). *DREB2C* functions to mediate the response of plants to heat

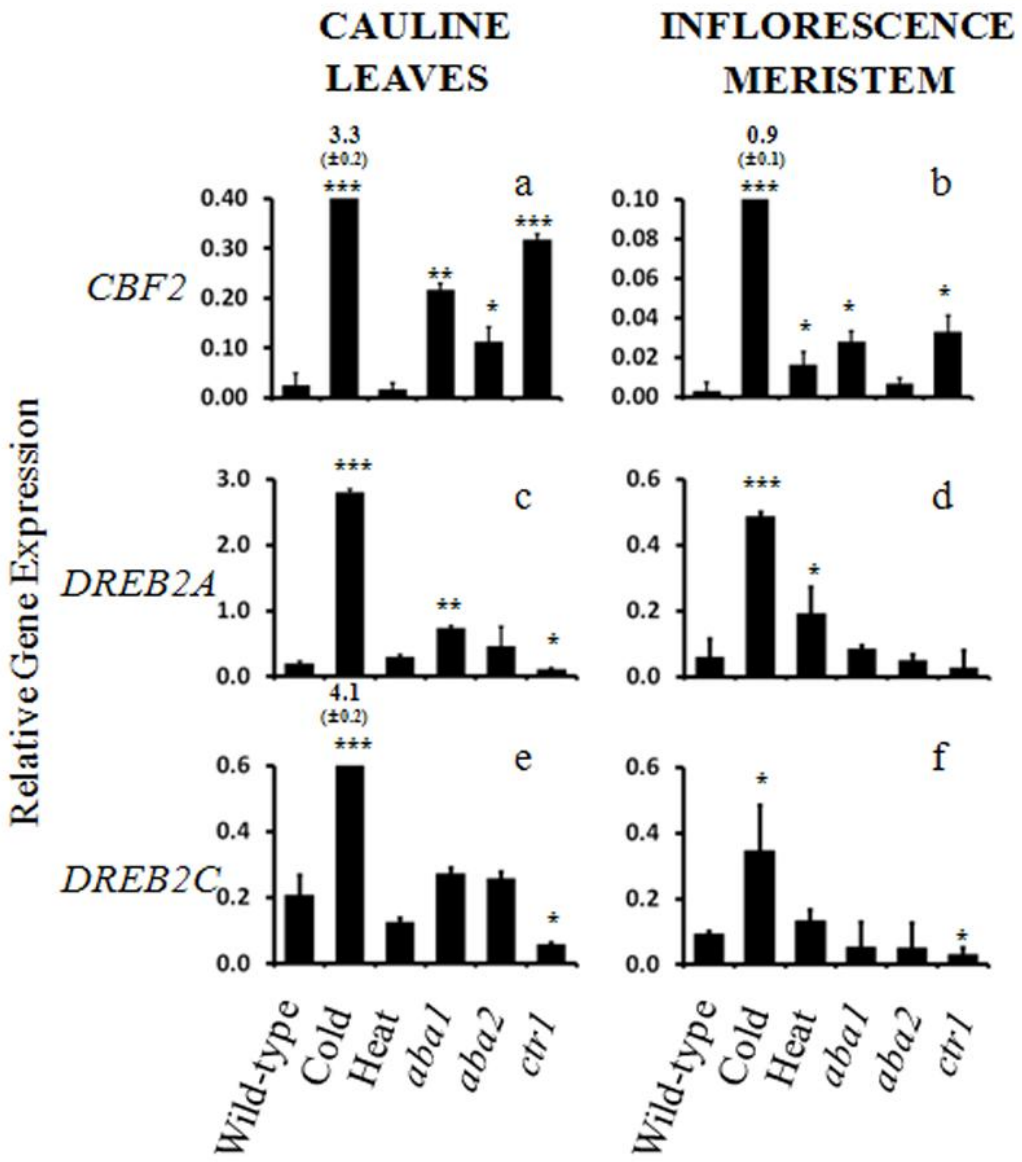

Fig. 4.3 Expression of ABA-independent transcription factors in Arabidopsis. Figures **a** through **f** represent the expression level of genes in cauline leaves and inflorescence meristems of reproductive stage plants. Wild-type, *aba1*, *aba2* and *ctr1* mutants were grown under identical conditions (22°C). For abiotic stress treatments a subset of wild-type plants were exposed to severe temperature stress (cold = 0°C, heat = 37°C) for 24 h. For a given gene (*CBF2*, *DREB2A*, *DREB2C*) and tissue (cauline leaves, inflorescence meristem) astericks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to wild-type plants grown at 22°C. Data correspond to mean and standard deviation (SD) of three biological replicates. RT-qPCR data were normalized to the expression of the internal reference gene *PP2A* (*At1g13320*). P-values determined according to a two-tailed studentized t-test (equal variance).

stress (Lim et al. 2007) and in the current study, we found *DREB2C* was upregulated 19.4-fold and 3.7-fold by cold stress. However, in both cauline leaves and inflorescence meristems exposed to heat stress, *DREB2C* expression was unaltered (Fig. 4.3e,f). In both *aba1* and *ctr1* mutants, *CBF2* was up-regulated in both cauline leaves and inflorescence meristems. With the exception of *DREB2A* expression in cauline leaves, *DREB2A* and *DREB2C* expression was unaffected in ABA-deficient backgrounds. However, in *ctr1* mutants, *DREB2A* or *DREB2C* expression was repressed in cauline leaves or inflorescence meristems (Fig. 4.3c,e,f).

4.4.4 Expression of genes involved in sugar signaling and metabolic stress

In recent years shared components of ABA and sugar signaling pathways (e.g. *KIN10/SnRK1.1* and *KIN11/SnRK1.2*) have emerged as central regulators of stress and energy signaling in plants and interact with transcription factors (ABI4, ABI5) mediating the response of plant cells to light and sugars (Acevedo-Hernandez et al. 2005; Baena-González et al. 2007; Bossi et al. 2009; Jossier et al. 2009). In the current study, *ABI4* and *ABI5* were upregulated in cauline leaves and inflorescence meristems exposed to cold stress (Fig. 4.4a,b,c,d). In cauline leaves exposed to heat stress, *ABI4* and *ABI5* levels were unaffected whereas in inflorescence meristems, both were up-regulated (Fig. 4.4a,b,c,d). Within the exception of inflorescence meristems exposed to heat stress, *KIN10* and *KIN11* expression was relatively unaffected by abiotic stress or within mutant backgrounds (Fig. 4.4e,f,g,h). The lack of change in *KIN10* or *KIN11* expression in the current study was not unexpected, as changes in KIN10/KIN11 activity occur primarily at a post-translational level (Fragoso et al. 2009).

Within *aba1* and *aba2* mutant backgrounds, *ABI4* and *ABI5* were differentially expressed. In *aba1* mutants, *ABI4* and *ABI5* transcripts were lower in cauline leaves, but unaltered in inflorescence meristems. However, in *aba2* mutants, *ABI4* expression was upregulated in both cauline leaves and inflorescence meristems, whereas *ABI5* expression was unaltered in these same organs. In cauline leaves and inflorescence meristems of *ctr1* mutants *ABI5* expression was unaltered relative to wild-type plants (Fig. 4.4c,d). However, in this same mutant, *ABI4* transcripts were up or down-regulated depending on the tissue sampled (Fig. 4.3 a,b). In response to abiotic stress or across mutant backgrounds, relative levels of *ABI5* transcripts within inflorescence meristems consistently exceeded those observed in cauline leaves, suggesting *ABI5* may be subject to a significant degree of developmental regulation (Fig. 4.4c,d).

4.4.5 MDC staining to detect autophagosomes during plant development and stress responses

In addition to assessing transcriptional differences in *ATG* or ABA response genes between source (autotrophic) and sink (heterotrophic) organs of *Arabidopsis thaliana*, we sought to identify cell types of reproductive organs characterized by elevated levels of autophagic activity during plant development or in response to abiotic stress. The autofluorescent dye monodansylcadaverine (MDC) has previously been characterized as a morphological marker of autophagy in Arabidopsis (Contento et al. 2005; Chen et al. 2009). However, the use of this marker has been focused on heterotrophic tissues such as suspension cells, protoplasts or seedling roots.

Under conditions conducive to autophagosome formation (e.g. carbon starvation, H_2O_2 treatment), the elongation zone and root tip of wild-type seedlings stained with

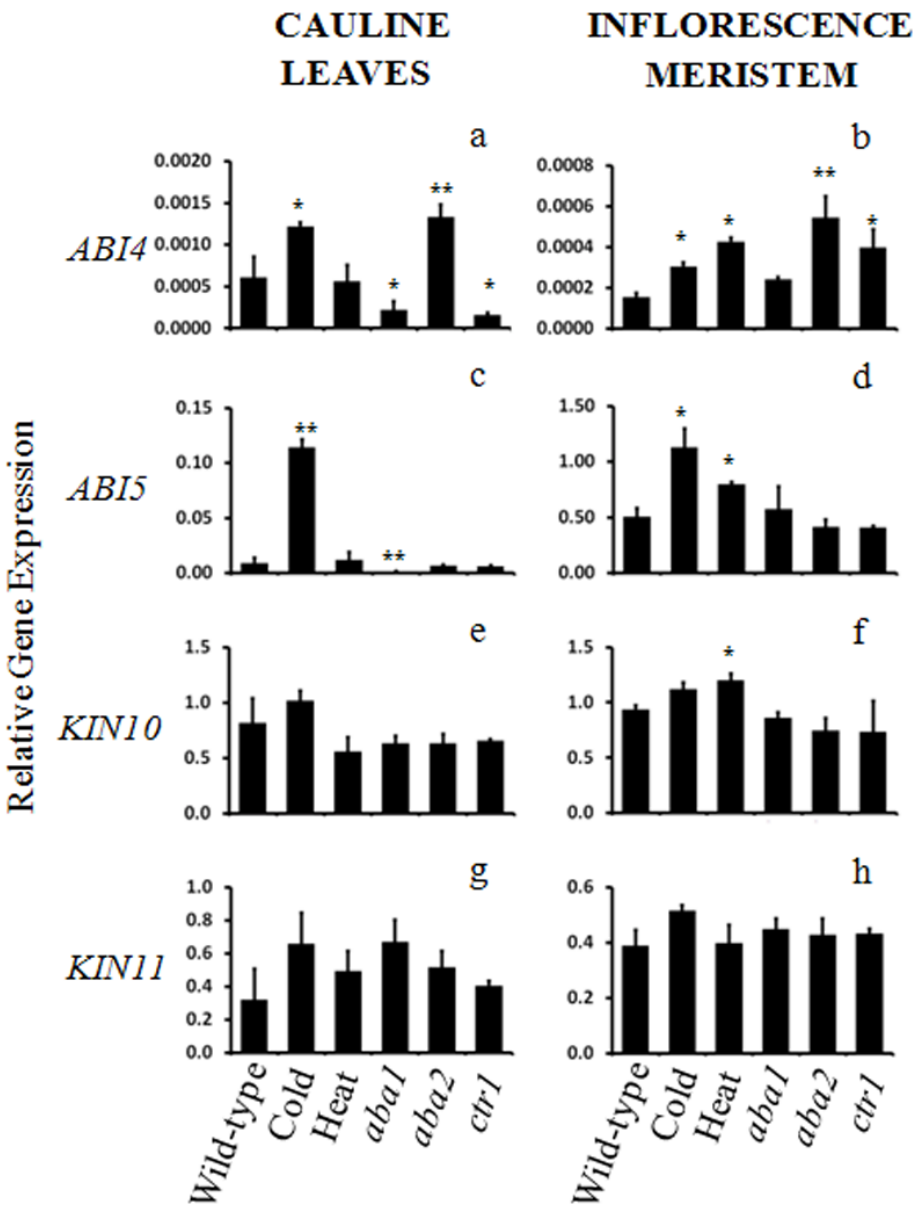

Fig. 4.4 Expression of *ABI4*, *ABI5*, *KIN10* and *KIN11* in Arabidopsis. Figures **a** through **h** represent the expression level of genes in cauline leaves and inflorescence meristems of reproductive stage plants. Wild-type (WT), *aba1*, *aba2* and *ctr1* mutants were grown under identical conditions (22°C). For abiotic stress treatments a subset of wild-type (WT) plants were exposed to severe temperature stress (cold = 0°C, heat = 37°C) for 24 h. For a given gene (*ABI4*, *ABI5*, *KIN10*, *KIN11*) and tissue (cauline leaves, inflorescence meristem) astericks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to wild-type (WT) plants grown at 22°C. Data correspond to mean and standard deviation (SD) of three biological replicates. RT-qPCR data were normalized to the expression of the internal reference gene *PP2A* (*At1g13320*). P-values determined according to a two-tailed studentized t-test (equal variance).

MDC (Fig. 4.5a) and contained numerous motile, spherical structures resembling autophagosomes (cytosol) and autophagic bodies (vacuole) described by Contento et al. (2005). For epidermal peels isolated from leaves exposed to carbohydrate starvation (darkness) for 48 hours, numerous motile autophagsomes were detected within guard cells and subsidiary cells (Fig. 4.5b). However, within developing seeds (4-8 days after pollination) motile autophagosomes were detected in seed coat cells containing expanded vacuoles and starch granules (Fig. 4.5b). During seed germination, aleurone cells would also stain prominently for autophagosomes or autophagic bodies (Fig. 4.5b).

In the current study we further evaluated whether cold and heat stress treatments used to assess the expression of *ATG* genes could modify autophagosome formation or activity within reproductive organs. However, in our hands, cold or heat stress treatments applied to intact plants (16 h light:8 h dark) for 24 h were insufficient to trigger morphological changes in autophagy within cauline leaves or developing seeds (data not shown). Therefore, we chose to focus on the ability of temperature stress to modulate autophagy previously induced within carbon-starved seedlings or cauline leaves. For dark-incubated seedlings held at 22°C autophagosomes were highly motile and evenly distributed throughout the cytoplasm of root cells. In dark-incubated seedlings exposed to cold (0°C) or heat stress (37°C) a notable increase in autophagosomes and overall fluorescence was observed (Fig. 4.6a). The tendency for MDC-stained structures to aggregate in response to temperature stress greatly hampered attempts at quantifying autophagosomes or autophagic bodies per cell. Nevertheless, the aggregation of autophagosomes in temperature-stressed cells provided evidence for the conversion of autophagosomes (cytoplasm) to autophagic bodies (vacuole) (Fig. 4.6a). In seedlings

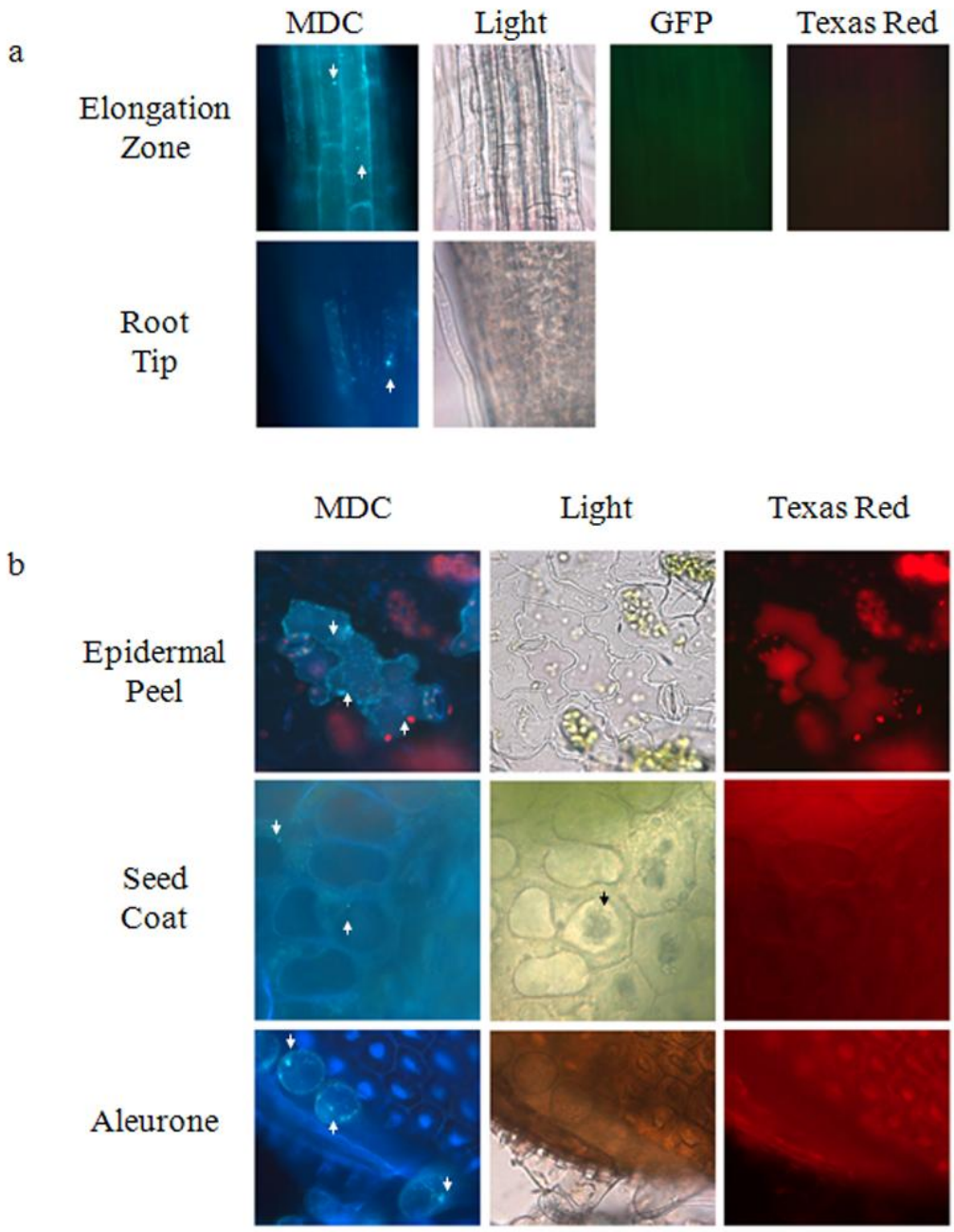

Fig. 4.5 Monodansylcadaverine (MDC) staining to detect autophagosomes in seedling roots, cauline leaves and seeds. **a** Autophagosomes were detected in the elongation zone and root tip of seedlings grown on sucrose-free medium. Within epidermal peels MDC stain localizes to guard cells and subsidiary cells. **b** Motile autophagosomes were observed in subsidiary cells of epidermal peels. During seed development autophagosomes were detected in seed coat cells. Aleurone cells associated with recently germinated seeds contain autophagosomes and autophagic bodies. White arrows mark autophagosomes. Black arrows mark starch granules.

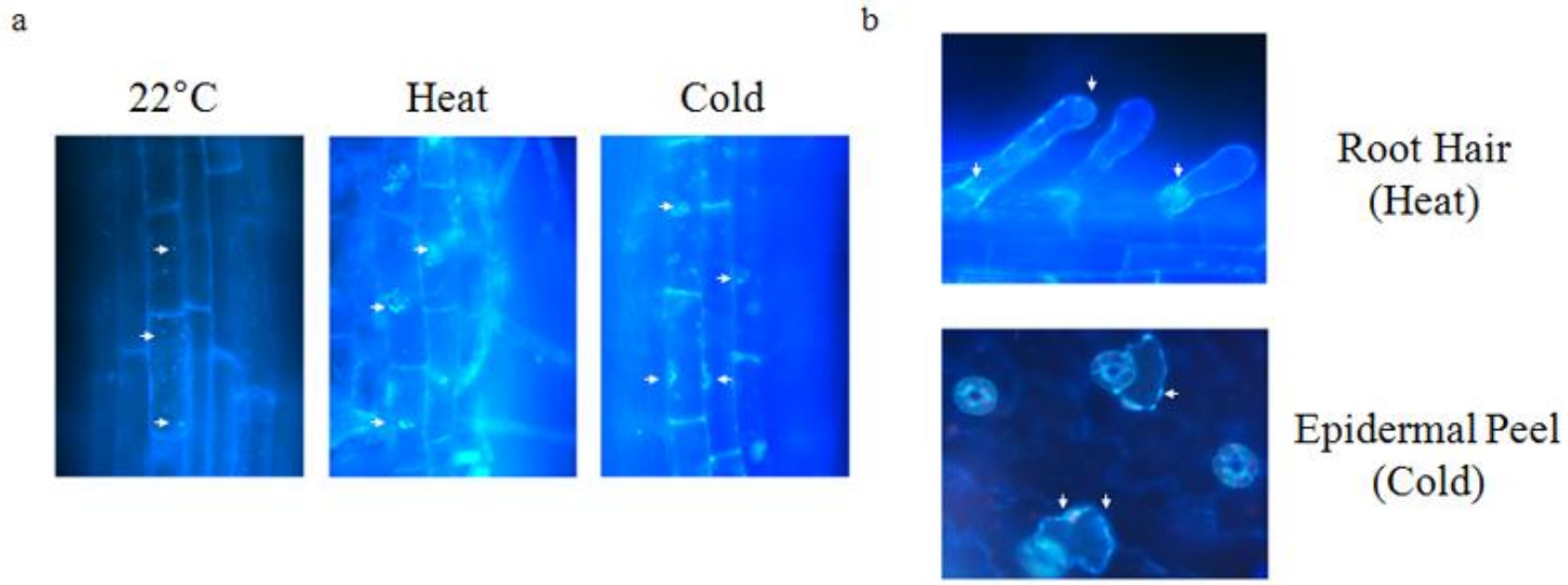

Fig. 4.6 Monodansylcadaverine (MDC) staining of carbon-starved seedlings and cauline leaves following cold and heat stress. **a** In seedling roots cold (0°C) and heat (37°C) stress treatment led to increases in MDC staining and changes in the localization of autophagosomes or autophagic bodies. **b** In seedling roots (top panel) exposed to heat stress, autophagosomes and autophagic bodies were detected in root hair cells. In cauline leaves exposed to cold stress (bottom panel), autophagosomes localized to the periphery of subsidiary cells.

exposed to heat stress, but not cold stress, autophagic bodies were also detected in root hairs (Fig. 4.6b). In detached, carbon-starved cauline leaves, low and high temperature extremes did not alter the number or motility of autophagsomes to the same extent as observed for temperature-stressed seedlings. However, in epidermal peels of cauline leaves exposed to cold stress, autophagosomes tended to aggregate near the periphery of subsidiary cells (Fig. 4.6b).

4.5 Discussion

4.5.1 Abiotic stress, ABA biosynthesis and signaling and the regulation of *ATG* genes

In the current study, we utilized cold and heat stress treatments, sugar signaling mutants and MDC staining to evaluate the transcriptional and morphological response of autophagy at the whole plant level. In response to cold and heat stress, several core *ATG* genes (e.g. *ATG8e, ATG18a*) were upregulated in both source and sink organs (Fig. 4.1). However, a key finding emerging from the current study surrounds the differential regulation of negative regulators of autophagy (e.g. *AtTOR, AtHVA22*) in response to abiotic stress, and in particular cold stress (Fig. 4.1; Fig. 4.2). Following cold stress, *AtHVA22* genes were highly expressed in both source and sink organs (Fig. 4.2) in a pattern which closely paralleled transcription factors (*CBF1-3*) associated with the cold-response pathway (Fig. 4.3). This observation was in agreement with results obtained by Chen et al. (2002). However, within these same tissues exposed to cold stress, *AtTOR* transcripts were either unaffected or down-regulated in source or sink organs, respectively (Fig. 4.1). Therefore, as characterized negative regulators of plant autophagy, *AtTOR* and members of *AtHVA22* gene family are differentially regulated by cold stress. In recent years, several studies have determined components of the CBF

response pathway, including *COLD-REGULATED* (*COR*) and *RESPONSIVE TO DEHYDRATION* (RD) genes, function to extend plant longevity and delay senescence, possibly through interfering with the metabolic regulation of senescence by sugars (Vogel et al. 2005; Masclaux-Daubresse et al. 2007; Sharabi-Schwager et al. 2009; Yang et al. 2011). In a subset of these studies, the delayed senescence phenotype of transgenic plants overexpressing CBF2 was accompanied by increased transcript levels of ABA biosynthesis and signaling genes, including several members of the *GRAM* and *AtHVA22* gene families, suggesting select family members may be direct transcriptional targets of the CBF response pathway. Given the established role of *HVA22/AtHVA22* genes as negative regulators of autophagy, future studies may be directed towards characterizing their role in freezing and chilling tolerance, where the relationship between sugars, ABA and ABA-independent signaling pathways remains unclear (Medina et al. 1999; Knight et al. 2004; Wingler and Roitsch 2008). In the current study, *CBF2* transcripts were elevated across source and sink organs of ABA-deficient/sugar signaling mutants (Fig. 4.3), indicating that in the absence of stress, endogenous ABA metabolism and signaling may antagonize the cold-response pathway.

In the present study we also sought to assess how genetic lesions affecting hormone biosynthesis and sugar signaling (*aba1, aba2, ctr1*) modified *ATG* and ABA-response genes in both source and sink plant organs. In response to cold and heat stress, *ABI4* and *ABI5* were upregulated within inflorescence meristems and cauline leaves in a manner similar to that of *ATG8e* and *ATG18a* (Fig. 4.1; Fig. 4.4). However, in ABA biosynthesis and sugar signaling mutants *ABI4* and *ABI5* expression patterns were often complex and showed no consistent relationship to *ATG* genes (Fig. 4.1; Fig. 4.4). For

example, *ABI4* was highly expressed in both cauline leaves and inflorescence meristems of *aba2-1* mutant. In the same tissues isolated from *aba1-1* mutant, *ABI4* expression was unaffected or down-regulated. With the exception of lower *ABI5* levels in cauline leaves of *aba1-1* mutant, *ABI5* was unaltered across hormone biosynthesis and sugar signaling mutants in the current study. However, *ABA1* and *ABA2* response loci fulfil disparate roles within stress (*ABA1*) and sugar (*ABA2*) signaling pathways (Cheng et al. 2002; Xiong et al. 2002a). Therefore, the elevated expression of *ABI4* in source and sink organs of the *aba2* mutant is of interest given previous efforts to characterize the genetic relationship between these ABA response loci in sugar signaling pathways (Dekkers et al. 2008).

In attempting to clarify the relationship between ABA response loci and *ATG* genes, previous studies determined *AtTOR* levels modulate the sensitivity of Arabidopsis seedlings to ABA or osmotic stress, with *AtTOR* knockdown lines displaying developmental arrest phenotypes reminiscent of plants overexpressing *ABI5* (Deprost et al. 2007). In the current study, *ABI5* transcripts were elevated within inflorescence meristems isolated from wild-type and mutant plants (Fig. 4.4). However, in contrast to results obtained by Menand et al. (2002), we did not detect elevated levels of *AtTOR* transcripts within inflorescence meristems (Fig. 4.1), although *AtTOR* and *ABI5* were regulated in opposing manner by cold and heat stress (Fig. 4.1;Fig. 4.4). Nonetheless, observations made in this study and others (Pourtau et al. 2004; Deprost et al. 2007; Liu and Bassham 2010) suggest the relationship between ABI5 and AtTOR in senescence, autophagy and sugar signaling requires further clarification.

During seedling development, Liu et al. (2009) reported ABA biosynthesis mutants display no apparent defects in autophagy and external applications of ABA fail to induce autophagy. However, at later stages of plant development ABA biosynthesis and signaling mutants display accelerated (*aba1, aba2*) or delayed (*abi5*) senescence phenotypes on medium containing high glucose in combination with low, but not high, nitrogen (Pourtau et al. 2004). Similar growth conditions induce core *ATG* genes in Arabidopsis (Wingler et al. 2009). In the current study, wild-type and mutants were grown in a soil-less potting mix under fertile, well-watered conditions. At the transcriptional level, we were unable to identify a clear relationship between ABA deficiency and the regulation of *ATG* genes (Fig. 4.1). In the future, assessing these same mutant backgrounds under nutrient deprived growth conditions may yield contrasting results. Combining loss-or-gain of *AtTOR* function lines with genetic backgrounds associated with the osmotic stress (ABA1, ATHK1) or sugar (ABI5, ABA2) branches of the ABA signaling pathway may also represent a genetically tractable means to dissect the role of ABA in autophagy and senescence.

4.5.2 Autophagic activity is preferentially localized within specific cell types

In the current study, we identified specific cell types of vegetative and reproductive organs characterized by elevated autophagic activity. The physiological relevance of autophagy in guard cells/subsidiary cells is currently unknown. However, as reactive oxygen species (ROS) serve as key secondary messengers in ABA signaling within guard cells (Kwak et al. 2003), and autophagy functions to degrade oxidized proteins (Xiong et al. 2007), an intriguing area of future research surrounds the

identification of cargo or signaling intermediates targeted for autophagic degradation in guard cells (Vanhee et al. 2011).

Seed coat and aleurone cells were also characterized by high levels of autophagic activity. In both monocots (*Zea mays)* and dicots (*Vigna mungo*), preceding studies have shown autophagic pathways or atypical autophagosomes function in the delivery of prolamins, zeins, starch granules and α-amylase to protein storage vacuoles (PSV) or lytic vacuoles (LV) (Toyooka et al. 2001; Reyes et al 2011). In Arabidopsis, several ABA-response proteins (ATI1, ATI2, TSPO, HVA22) associated with autophagic pathways are expressed at high levels during seed development and dessication (Guo and Ho 2008; Guillaumot et al. 2009; Honig et al. 2012). Antagonist interactions between ABA and GA have also been shown to mediate starch degradation in outer integuments of developing seeds (Kim et al. 2005; Arsovski et al. 2010) and further mediate vacuolation of aleurone cells during seed germination (Bethke et al. 2007).

Taken together the results of the current study have provided new information on the transcriptional regulation of autophagy during plant development and in response to environmental stress. We have further identified specific cell types characterized by elevated levels of autophagy, which may aid in directing future molecular and physiological studies of autophagy at the whole plant level.

Table 4.1S List of oligonucleotide sequences utilized for RT-qPCR analysis of gene expression in wild-type plants and mutants deficient in hormone biosynthesis and sugar signaling.

Gene	AGI Code		Oligo Sequence
ATG7	*At5g45900*	Forward	5'-ACCGCTTGCTCTGAAACCGTGATA-3'
		Reverse	5'-TCGTCTTCCCAGTCGAGGTTGAAT-3'
ATG8e	*At2g45170*	Forward	5'-GACGCGGAATTTCGATTTGGGTGT-3'
		Reverse	5'-TTCTCCCTGATCCTTCCAGCTTCA-3'
ATG18a	*At3g62770*	Forward	5'-AAGTTCGGATCGAGCACTACGCTT-3'
		Reverse	5'-ACCTCTTGACGCAAGGTACCATCA-3'
AtTOR	*At1g50030*	Forward	5'-TCTTCGGTTCTCGACGCATCATGT-3'
		Reverse	5'-AGATTGCGACGAGGTAGACA-3'
AtHVA22a	*At1g74520*	Forward	5'-GGTGTTTGTTAACCCTCGGAGCAT-3'
		Reverse	5'-TCAGCCCGGCTTAAGATCTTCTCA-3'
AtHVA22d	*At4g24960*	Forward	5'-AACAGTTCAAGAAACACGGCGTCC-3'
		Reverse	5'-ATGGGACCACCACCAAGACCATTA-3'
AtHVA22e	*At5g50720*	Forward	5'- ACGGCATCCTCAAACCTAAGGTAG-3'
		Reverse	5'-CCAACAGTGAAGCCCTCTTGTGT-3'
CBF2	*At4g25470*	Forward	5'-TGTCTCAATTTCGCTGACTCGGCT-3'
		Reverse	5'-AAGACCATGAGCATCCGTCGTCAT-3'
DREB2A	*At5g05410*	Forward	5'-ACGTGACCTAAATGGCGACGATGT-3'
		Reverse	5'-ACTTTGTTGACTCTCGGGCCTGTA-3
DREB2C	*At2g40340*	Forward	5'-AGGGAGACTTTGGATGCTTGGTTG-3'
		Reverse	5'-GGGTGTCTATCCACTTGATACTGC-3'
ABI4	*At2g40220*	Forward	5-CTTCGTTTCATCATGAGGTGGCGT-3'
		Reverse	5'-AACCAACGGCGGTGGATGAGTTAT-3'
ABI5	*At2g36270*	Forward	5'-TTGGTGAAGGCTGGTGTGGTTAGA-3'
		Reverse	5'-ATAGCTTGACCCGGGAATGAAGGA-3'
KIN10	*At3g01090*	Forward	5'-ATCGTTTCCGTGCCTCTAGTGGTT-3'
		Reverse	5'-ATTCCATCAGTCCTGGAAGCCGAT-3'
KIN11	*At3g29160*	Forward	5'-TTATCTTGCTGTCTCTCCACCGGA-3'
		Reverse	5'-GGAACACGGAACCGGTTATCCAAT-3'
PDF subunit PP2A	*At1g13320*	Forward	5'-TAACGTGGCCAAAATGATGC-3'
		Reverse	5'-GTTCTCCACAACCGCTTGGT-3'
PDF subunit PP2A	*At1g13320*	Forward	5'-CATGTTCCAAACTCTTACCTGCGG-3'
		Reverse	5'-TGGGTCTTCACTTAGCTCCACCAA-3'

5.0 Transcriptional response of abscisic acid (ABA) metabolism and transport to cold and heat stress applied at the reproductive stage of development in *Arabidopsis thaliana*

[Baron KN, Schroeder DF, Stasolla C (2012) Plant Sci 188:48-59]. Permission to include the published manuscript in the thesis has been obtained from the publisher/copyright holder.

5.1 Abstract

The phytohormone abscisic acid (ABA) plays an important role in developmental processes in addition to mediating plant adaptation to stress. In the current study, transcriptional response of 17 genes involved in ABA metabolism and transport has been examined in vegetative and reproductive organs exposed to cold and heat stress. Temperature stress activated numerous genes involved in ABA biosynthesis, catabolism and transport; however, several ABA biosynthesis genes (*ABA1*, *ABA2*, *ABA4*, *AAO3*, *NCED3*) were differentially expressed (up- or down-regulated) in an organ-specific manner. Key genes (*CYP707A*s) involved in ABA catabolism responded differentially to temperature stress. Cold stress strongly activated ABA catabolism in all organs examined, whereas heat stress triggered more subtle activation and repression of select *CYP707A* genes. Genes involved in conjugation (*UGT71B6*), hydrolysis (*AtBG1*), and transport (*ABCG25*, *ABCG40)* of ABA or ABA glucose ester responded to temperature stress and displayed unique organ-specific expression patterns. Comparing the transcriptional response of vegetative and reproductive organs revealed ABA homeostasis is differentially regulated at the whole plant level. Taken together our

findings indicate organs in close physical proximity undergo vastly different transcriptional programs in response to abiotic stress and developmental cues.

5.2 Introduction

In plants, the hormone abscisic acid (ABA) is often associated with inhibition of growth during abiotic or biotic stress conditions and also functions as a mediator of key phase transitions throughout the plant lifecycle (Finkelstein et al. 2002; Umezawa et al. 2010). However, several lines of evidence indicate basal levels of ABA metabolism and signaling exert a promotive effect on plant growth, development and physiology. For example, ABA-deficient mutants of *Arabidopsis thaliana* and tobacco (*Nicotiana plumbaginifolia*) display vegetative and reproductive phenotypes (e.g. impaired shoot growth, unfertilized ovules, aborted embryos) that can be rescued through exogenous applications of ABA (Cheng et al. 2002; Frey et al. 2004; LeNoble et al. 2004; Barrero et al. 2005). Low concentrations of ABA also stimulate root growth and promote maintenance of stem cells (De Smet et al. 2006; Zhang et al. 2010). ABA accumulates within inflorescence and floral meristems at the reproductive stage of development and may function in regulating assimilate distribution within reproductive organs (Yarrow et al. 1988; Peng et al. 2006). Hormone profiling studies further revealed reproductive structures (flowers, siliques) accumulate substantial quantities of ABA and related metabolites (Priest et al. 2006; Kanno et al. 2010). At present, very little is known as to how ABA promotes the growth of vegetative and reproductive organs in the absence of stress.

For economically important crop species ABA holds a key role in directing physiological processes that improve the stress tolerance of reproductive structures

exposed to cold, heat and drought (Liu et al. 2005; Barnabás et al. 2008; Thakur et al. 2010). Molecular studies in rice (*Oryza sativa*) and wheat (*Triticum aestivum*) also revealed unique cellular and organ-level aspects of ABA homeostasis that distinguish stress-tolerant and stress-sensitive germplasm at the reproductive stage of development (Oliver et al. 2007; Ji et al. 2011). From these studies, excessive ABA accumulation within reproductive structures was proposed to negatively correlate with abiotic stress tolerance. However, existing studies also provide evidence that the beneficial or detrimental role(s) assigned to ABA accumulation during reproductive development are heavily dependent upon plant phenology and physiological context (Yarrow et al. 1988; Yang et al. 2004; Liu et al. 2005; Nitsch et al. 2009). During the course of studying reproductive phenotypes associated with ABA- and stress-responsive GRAM (**G**lucosyltransferases, **Ra**b-like GTPase activators, and **m**yotubularins) genes in Arabidopsis, we sought to determine whether rapidly growing reproductive organs (inflorescence meristems, developing siliques) displayed unique features with respect to ABA metabolism and transport when compared with adjacent vegetative tissues (cauline leaves) (Jiang et al. 2008). A large proportion of transcriptional studies examining ABA biosynthesis and catabolism have been conducted utilizing tissues associated with classical ABA responses such as seed dormancy and germination, seed maturation, developmental arrest of seedlings, or the response of vegetative tissues to abiotic stress (Nambara and Marion-Poll 2005). However, the molecular understanding of ABA metabolism and transport at the whole plant level remains incomplete in spite of abundant physiological evidence indicating ABA is a mobile signaling molecule capable

of facilitating communication at the cellular, organ, whole plant and community levels (Zeevaart et al. 1999; Umezawa et al. 2010; Wilkinson and Davies 2010).

Recent studies of ABA metabolism and homeostasis in the model plant *Arabidopsis thaliana* have led to identification of several novel proteins (UGT71B6, AtBG1, ABCG25, ABCG40) which modify endogenous ABA levels or mediate the active transport of ABA between plant cells (Fig. 5.1) (Lee et al. 2006; Priest et al. 2006; Kang et al. 2010; Kuromori et al. 2010). However, there is currently little or no molecular information describing how established pathways responsible for *de novo* ABA biosynthesis or catabolism are integrated with pathways mediating ABA transport or homeostasis. Understanding how ABA metabolism and transport are regulated at the whole plant level serves to enhance the understanding of ABA action in plant physiology (Nambara and Marion-Poll 2005). In the present study, we analyzed the expression profile of Arabidopsis genes involved in ABA metabolism, transport and homeostasis in vegetative and reproductive organs at normal temperature and following cold and heat stress. Our results indicate key genes involved in ABA metabolism, transport and homeostasis are subject to a substantial degree of developmental regulation. Moreover, our findings provide evidence reproductive organs undergo contrasting transcriptional programs regulating ABA homeostasis in response to abiotic stress. Preceding studies revealed floral organs and early reproductive structures are particularly vulnerable to environmental stress (cold, heat, drought) and play a key role in the determination of crop yields (Liu et al. 2005; Barnabás et al. 2008; Thakur et al. 2010). In this regard, the current study offers new insight into how ABA metabolism and response operate during

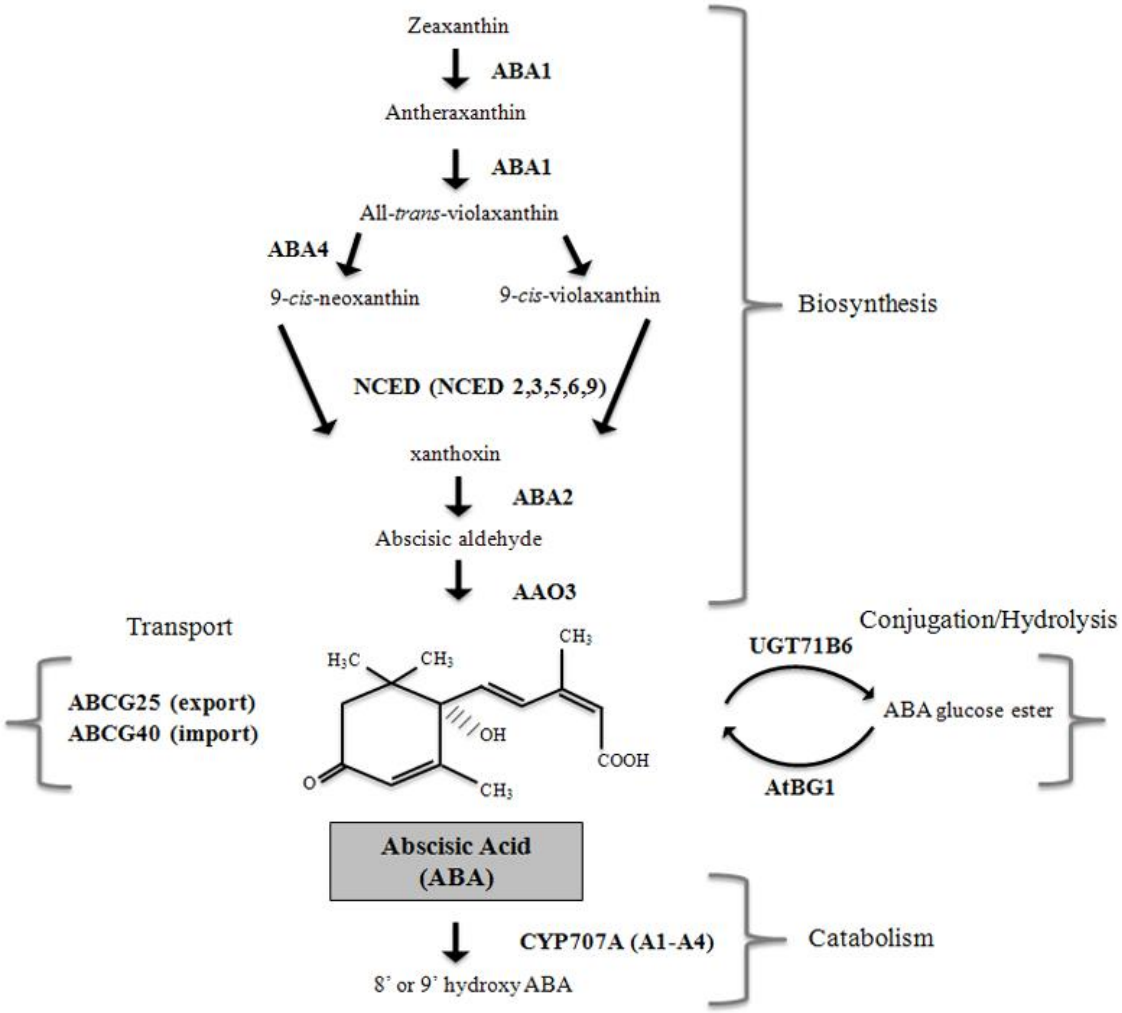

Fig. 5.1 Schematic representation of key genes involved in regulation of ABA homeostasis in *Arabidopsis thaliana*. ABA1 = zeaxanthin epoxidase (TAIR ID: AT5G67030.1), ABA2 = short chain alcohol dehydrogenase (TAIR ID: AT1G52340.1), ABA4 = neoxanthin synthase (TAIR ID: AT1G67080.1), NCED = nine-*cis*-epoxy carotenoid dioxygenase (NCED) (TAIR ID: AT4G18350.1) (TAIR ID: AT3G14440.1) (TAIR ID: AT1G30100.1) (TAIR ID: AT3G24220.1) (TAIR ID: AT1G78390.1), AAO3 = abscisic aldehyde oxidase (TAIR ID: AT2G27150.1), CYP707A = 8' or 9' ABA hydroxylase cytochrome P450 (TAIR ID: AT4G19230.1) (TAIR ID: AT2G29090.1) (TAIR ID: AT5G45340.1) (TAIR ID: AT3G19270.1), AtBG1 = ABA specific β-glucosidase (TAIR ID: AT1G52400.1), UGT71B6 = ABA specific glucosyltransferase (TAIR ID: AT3G21780.1), ABCG25 = ATP-binding cassette (ABC) transporter (TAIR ID: AT1G71960.1), ABCG40 = ATP-binding cassette (ABC) transporter (TAIR ID: AT1G15520.1).

plant development and in response to abiotic stress. This information can in turn be utilized towards enhancing the abiotic stress tolerance of reproductive development in plants.

5.3 Materials and methods

5.3.1 Plant material and growth conditions

Arabidopsis thaliana (Columbia ecotype) seeds were surface sterilized (70% EtOH, 0.5% Triton) and placed on sterile agar plates containing half strength MS medium. Plates were stratified for 2 d at 4°C in the darkness after which plates were placed under a light bar at a constant temperature of 22 ± 1.5°C and providing 140 ± 20 μmol $m^{-2}s^{-1}$ from cool white fluorescent lamps. Following germination, seedlings (4 true leaf stage) were transplanted in 8.5cm^2 square pots containing media (LA4 mix, aggregate plus, SunGro Sunshine, Seba Beach, AB). After transplanting, flats of seedlings were transferred to identical growth chambers (Model GC-20, Econair, Winnipeg, MB) providing constant 22°C ± 1.0°C on a 16 h (day):8 h (night) cycle and 70% relative humidity. For RT-qPCR analysis, general histology and ABA analysis following temperature treatments, tissues (cauline leaves, inflorescence meristems, developing siliques) were sampled at a stage of development corresponding to growth stages 6.3 – 6.5 (Boyes et al. 2001). At this stage (mid-flowering) plants generated an established primary inflorescence with ≥ 3 axillary inflorescences. Rosette and cauline leaves displayed no visual signs of senescence.

5.3.2 Stress treatments

For temperature treatments, either cold (0.0 ± 2.0°C) or heat (37.0 ± 2.0°C) stress was applied for 24 h beginning at subjective sunset. Prior to imposition of temperature regimes, based on previous studies examining temperature-induced reproductive failure in *Arabidopsis thaliana* (Lee and Lee 2003; Warner and Erwin 2005), plants were transferred to identical growth cabinets and ramped from 22°C to target temperatures (0.0 or 37.0°C) at a rate of 2.0°C/h. One day (24 h) prior to application of temperature treatments wild-type plants grown at 22°C were simultaneously removed from the growth chamber and inflorescence meristems marked with thread to track acceleration or delays in flowering associated with temperature stress. Marked plants were returned to growth chambers to enable reacclimation prior to being transferred to cold or heat stress.

For general histology, RT-qPCR studies and analysis of ABA content, inflorescence meristems were collected and pooled from a minimum of three plants. For RT-qPCR and ABA analysis 4-6 developing siliques (5-10 days post anthesis in subtending positions) and 2-3 cauline leaves per plant were sampled from primary and axillary inflorescences. Some cold and heat stressed plants were also returned to ambient conditions to gauge the severity of temperature treatments.

5.3.3 General histology

For general histology inflorescence meristems were vacuum-infiltrated and fixed overnight (4°C) on a rotator in a mixture of cold 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.05 M phosphate buffer (pH 6.8). Samples were then dehydrated with methyl cellosolve (2-methoxyethanol) and two repeats of 100% ethanol, followed by infiltration and embedding in Historesin (Leica Microsystems, Concord, ON, Canada).

Sections of inflorescence meristems (3 μM) were then stained with Periodic Acid Schiff (PAS) for total carbohydrates, and counterstained with toluidine blue (TBO) for general histological organization.

5.3.4 ABA measurements

The ABA concentration of cauline leaves, inflorescence meristems and developing siliques was measured using the Phytodetek competitive ELISA kit (Agdia, Elkhardt, IN, USA). Plants exposed to cold and heat stress for 24 h were sampled at the same time of day. Similar tissues were collected from plants grown under an ideal (22°C) temperature regime. Reproductive organs were first ground in liquid nitrogen, freeze-dried and dry weights determined. Tissue analyzed for ABA content represents pooled tissue combined from at least three plants. After determination of dry weights, powdered tissues were extracted overnight in 1 mL of ABA extraction buffer (methanol containing 100 mg/L butylated hydroxyl toluene, 0.5 g/L citric acid monohydrate) using gentle agitation. Samples were then centrifuged at 2500 xg and supernatants dried under a stream of N_2 gas. Pelleted samples were then re-extracted with an additional 1 mL of ABA extraction buffer, combined with initial dried extracts, and dried under N_2 gas. Dried extracts were re-suspended in 100 μL of methanol followed by addition of 900 μL of 1x Tris-TBS buffer (supplied with Phytodetek kit). For ABA ELISAs 5-20 μL of the final extract was diluted in 1x TBS to achieve a final volume of 100 μL and analyzed according to the Phytodetek protocol. ABA concentrations were calculated as ng per g DW.

5.3.5 RNA isolation and RT-qPCR analysis

Total RNA was extracted from cauline leaves, inflorescence meristems and developing siliques using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) with genomic DNA removed through on-column digestion using an RNase-free DNase Kit (Qiagen) according to the manufacturer's protocol. An aliquot of total RNA was subsequently analyzed on a Nano-drop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Depending on the tissue 1-5 μg of total RNA was utilized to synthesize first-strand cDNA using a Maxima^{TM} First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada) for RT-qPCR. Following synthesis cDNA concentration was determined and cDNA diluted to an appropriate volume. Quantitative RT-PCR studies were then carried out using the Sso Fast™ Evagreen® SuperMix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) with a final reaction volume of 20 μL (10 μL master mix, 1 μL cDNA, 9 μL primer mix). In a single experiment, each sample was assayed in triplicate and expression levels were calculated relative to the internal reference gene phosphatase 2A (*PP2A = At1g13320*) subunit (Czechowski et al. 2005). Triplicate PCR's (technical) were conducted on three separate cDNAs (biological) with each cDNA representing RNA extracted from pooled tissue collected from at least three plants. During data analysis wells producing abnormal amplification plots or dual melting curves were eliminated from subsequent analyses. RT-qPCR data was analyzed according to the comparative C_t ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak 2008), where ΔC_T is calculated for each sample as the difference between the *Ct* of a gene of interest and the *Ct* of a reference gene (e.g. *PP2A*). Oligonucleotide sequences used for RT-qPCR studies provided in Table 5.1S.

5.4 Results

5.4.1 Cold and heat stress negatively impact reproductive development

In the current work Arabidopsis plants (Col-0) were gradually exposed to severe temperature extremes with the intention of negatively impacting reproductive development (Lee and Lee 2003; Warner and Erwin 2005). At a macroscopic level cold and heat stress treatments caused visible changes in the pigmentation of inflorescence meristems, flowers and developing siliques (Fig. 5.2a-c). For plants exposed to heat stress, growth of stamen filaments was severely impaired preventing stamens from pollinating the stigma. At a structural level imposition of cold and heat stress caused several abnormalities to reproductive organs (Fig. 5.2d-l). These included early signs of vacuolation and collapse within microspores (Fig. 5.2h), abnormal pollen sacs which failed to accumulate starch products (Fig. 5.2i), and differences in timing of tapetum degeneration (Fig. 5.2j,k). Collectively, these macroscopic and histological observations provided evidence that temperature regimes were severe enough to trigger visible symptoms of injury that would negatively impact male and female gametophyte development. However, the imposed temperature treatments were not lethal. After tissue sampling all plants were returned to 22°C and allowed to recover. After 2-3 weeks additional growth/re-growth all plants subjected to non-destructive harvests were able to produce viable fruits (not shown). This was consistent with comparable studies evaluating the anatomical response of *Arabidopsis thaliana* plants exposed to abiotic stress at the reproductive stage of development (Kim et al. 2001; Sun et al. 2004). Taken together these observations indicate that changes in gene expression and ABA

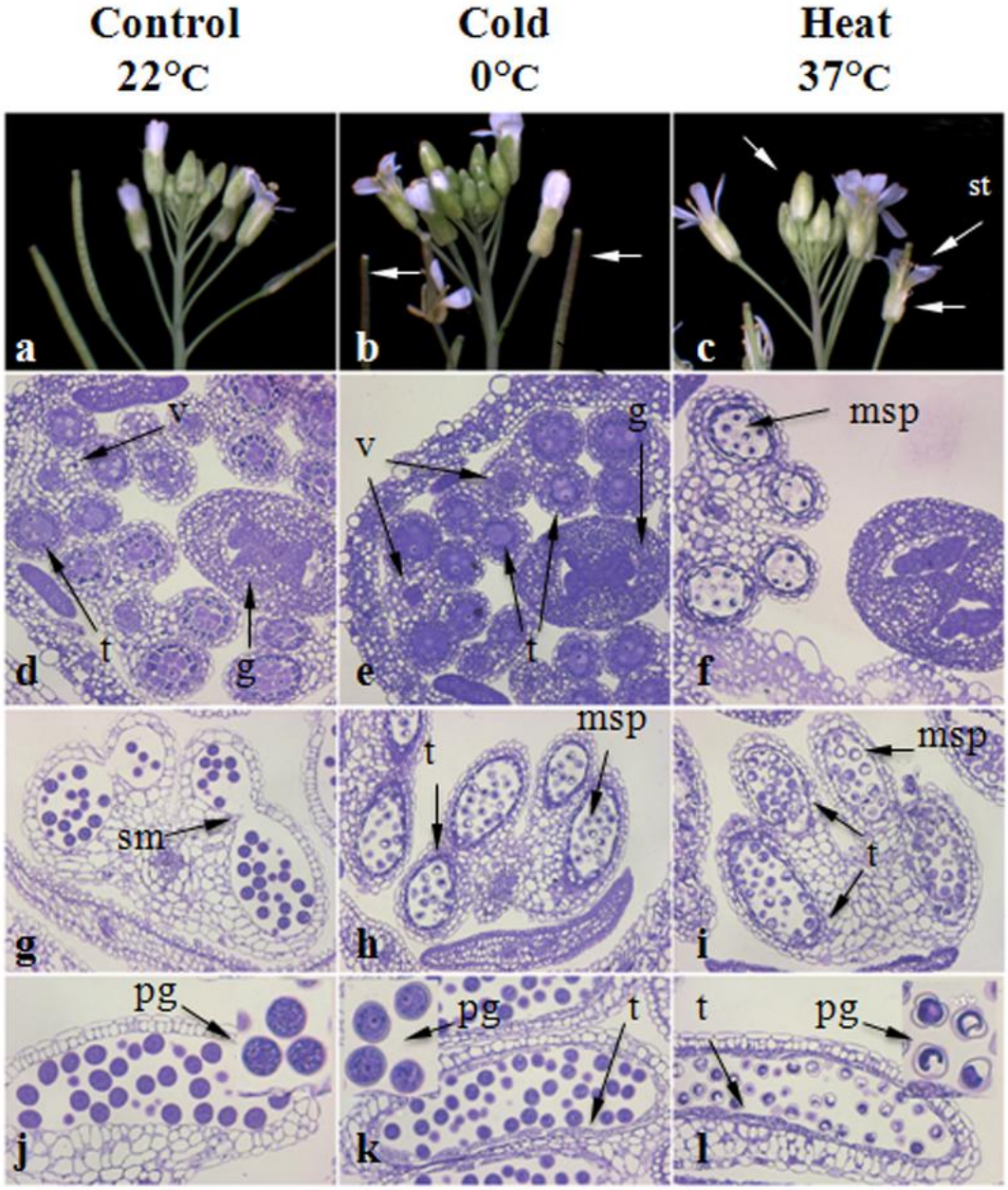

Fig. 5.2 Cold and heat stresses disrupt the reproductive development of *Arabidopsis thaliana*. **a** Healthy inflorescence meristem, flowers and developing siliques. **b** In response to cold stress, developing siliques display visible signs of anthocyanin accumulation (white arrows). **c** Inflorescence meristems and flowers exposed to heat stress were noticeably yellowed (white arrows). Stamen filaments (st) of heat-stressed plants were unable to pollinate the stigma. **d-l** Inflorescence meristems sampled for structural analysis contained anthers at various stages of development beginning with the emergence of stamen primordia (Stage 1) through to senescence of stamens (Stage 14). Anther staging corresponds to Sanders et al. (1999). **d** Transverse section of healthy

Figure 5.2 (continued) stage 6 anther and adjacent gynoecium. Vascular region (v), tapetum (t) and gynoecium (g). **e** Stage 6 anther and gynoecium exposed to cold stress. Vascular region (v), tapetum (t) and gynoecium (g) are prominently stained. **f** As early as stage 9, anthers and microspores (msp) exposed to heat stress display signs of vacuolation. **g** Healthy stage 12 anther showing initial breakage of septum (sm) during anther dehiscence. **h** Stage 10 anther exposed to cold stress. Tapetum (t) is prominently stained. Developing microspores (msp) display signs of collapse. **i** Stage 10 anther exposed to heat stress. Pollen sacs are abnormally shaped and microspores (msp) fail to accumulate carbohydrates. Tapetum (t) is weakly stained or degenerated. **j** Within a healthy stage 12 anther, the tapetum has degenerated. Pollen grains (pg) are uniform with cytoplasm dispersed throughout the cell. **k** Stage 12 anther exposed to cold stress. The tapetum (t) has not degenerated. Pollen grains are comparable in size to pollen isolated from healthy plants. However, in mature pollen grains (pg), nuclei remain prominent. **l** Stage 12 anther exposed to heat stress. The tapetum (t) has not degenerated. Many pollen grains (pg) have become vacuolated or collapsed. At earlier stages of anther development (see Fig. 5.2f,h) cold and heat stress resulted in similar morphological changes in the structure of microspores (msp).

levels reported in the current study were associated with non-lethal levels of abiotic stress capable of negatively impacting plant fertility.

5.4.2 Expression analysis of ABA biosynthesis genes (*ABA1, ABA2, ABA4, AAO3*)

To investigate spatial patterns of ABA metabolism and homeostasis in response to abiotic stress quantitative real-time PCR (RT-qPCR) was used to analyze key genes involved in ABA biosynthesis (Fig. 5.1). In cauline leaves and inflorescence meristems expression of several ABA biosynthesis genes (*ABA1, ABA2, AAO3*) was strongly activated by cold and heat stress (Fig. 5.3). However, in developing siliques, many of these same genes were differentially expressed (up or down-regulated) as a consequence of the imposed stress (Fig. 5.3). In cauline leaves and inflorescence meristems, cold and heat stress appeared to preferentially activate select ABA biosynthesis genes over others. For example, *ABA1* and *AAO3* were highly responsive to cold stress whereas *ABA2* and *ABA4* appeared more responsive to heat stress (Fig. 5.3).

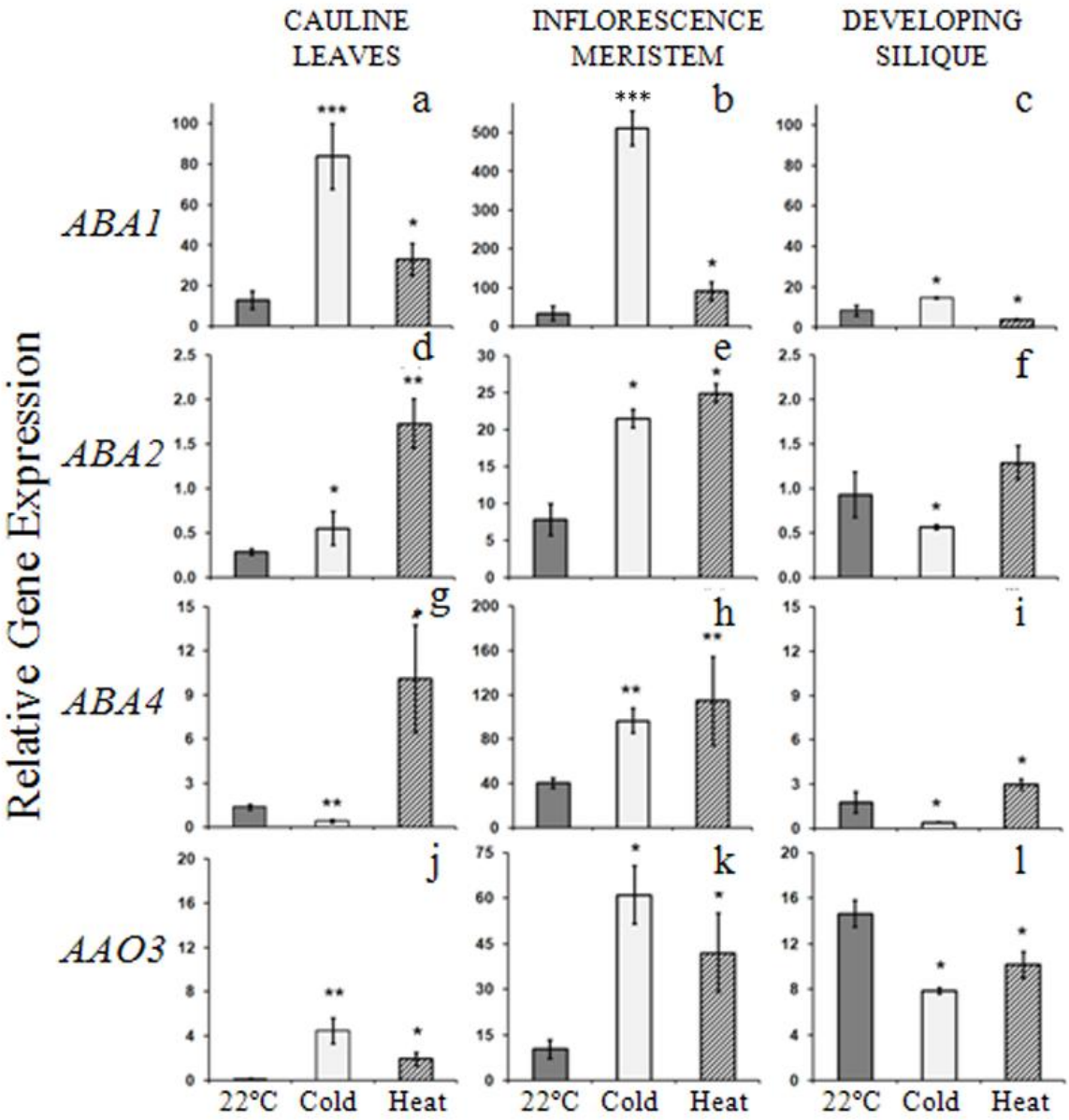

Fig. 5.3 Expression of genes involved in abscisic acid (ABA) biosynthesis. Figures **a** through **l** represent the expression level of genes in vegetative and reproductive organs under ideal growing conditions (22°C) or in response to severe temperature stress (cold = 0°C, heat = 37°C). For a given gene (*ABA1, ABA2, ABA4, AAO3*) and tissue (cauline leaves, inflorescence meristem, developing silique) astericks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to plants grown at 22°C. Data correspond to mean and standard deviation (SD) of three biological replicates. RT-qPCR data were normalized to the expression of the internal reference gene PP2A (*At1g13320*). Significant developmental patterns (22°C) of gene expression and associated P-values are discussed in text. P-values determined according to a two-tailed studentized t-test (equal variance). Note the relative gene expression scale for inflorescence meristems is higher than cauline leaves and developing siliques.

For several ABA biosynthesis genes (*ABA1*, *ABA2*, *ABA4*), higher relative levels of gene expression were consistently observed in inflorescence meristems over cauline leaves and developing siliques (Fig. 5.3, note altered scales). Considering plants grown at 22°C, *ABA2* expression levels were 27.3-fold ($P < 0.05$) and 8.4-fold ($P < 0.05$) higher in inflorescence meristems relative to cauline leaves and developing siliques, respectively (Fig. 5.3d-f). Similar developmental patterns of expression were observed for *ABA1* and *ABA4* (Fig. 5.3a-c,g-i). However, relative expression levels of *AAO3* were consistently higher in reproductive organs (inflorescence meristem, developing silique) relative to vegetative tissues (cauline leaves) (Fig. 5.3j-l).

5.4.3 Expression analysis of nine-*cis*-epoxycarotenoid dioxygenase (*NCED*) genes involved in ABA biosynthesis

Cleavage of 9-*cis*-xanthophylls to xanthoxin represents a key step in ABA biosynthesis in plants and is mediated by a small family of nine-*cis*-epoxycarotenoid dioxygenase genes in *Arabidopsis thaliana* (Fig. 5.1) (Tan et al. 2003). In the current study three NCED genes (*NCED-2,5,6*) were coordinately activated in cauline leaves exposed to cold stress (Fig. 5.4a,g,j). In contrast, these exact same NCED family members were repressed in cauline leaves following heat stress (Fig. 5.4a,g,j). Within inflorescence meristems the expression of these genes was relatively unaffected by either stress (Fig. 5.4b,h,k). However, in developing siliques, these same NCED family members were responsive to temperature stress (Fig. 5.4c,i,l). Notably, two genes (*NCED5,6*) repressed by heat stress in cauline leaves were activated by heat stress in developing siliques (Fig. 5.4). *NCED3*, often considered rate-limiting for ABA biosynthesis in response to abiotic stress, was also differentially expressed depending

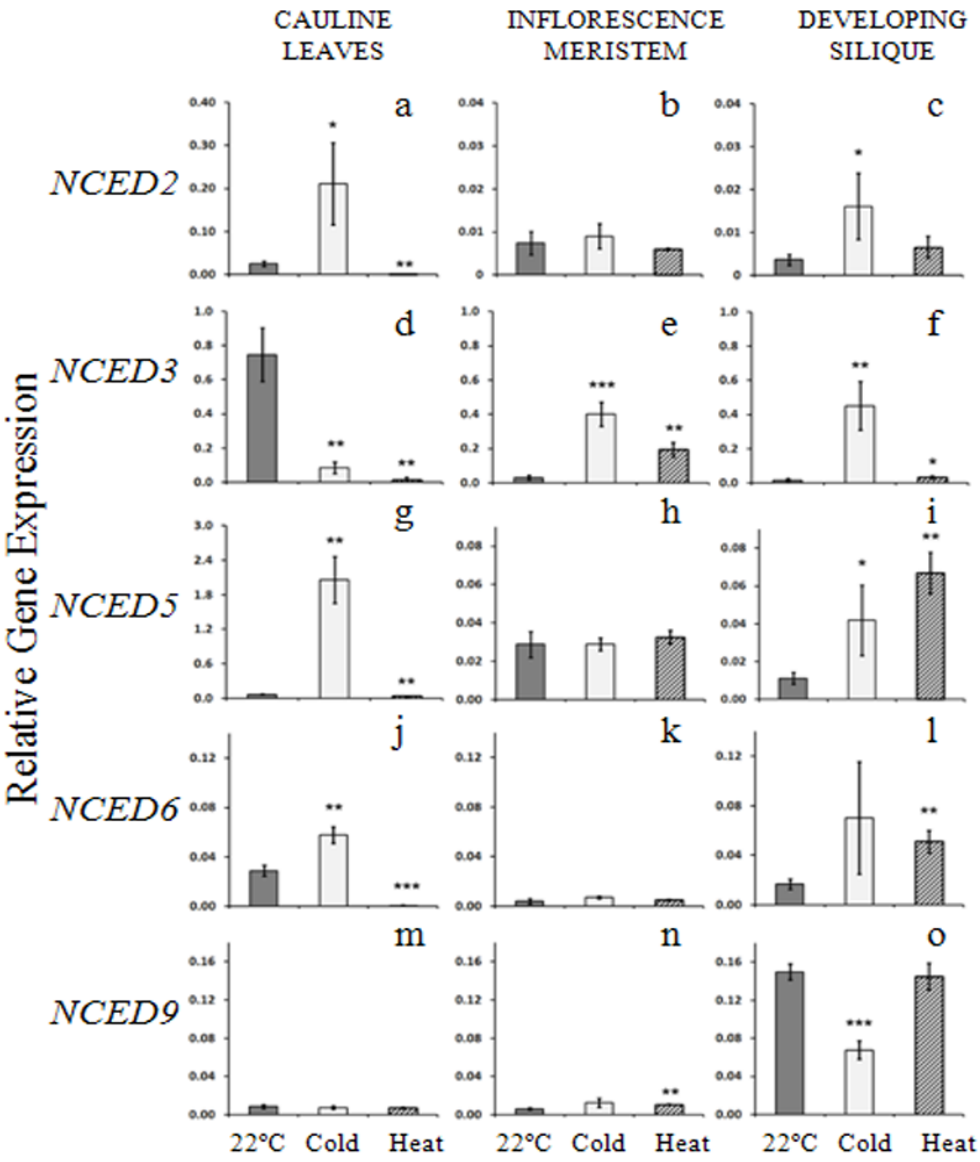

Fig. 5.4 Expression of nine-*cis*-epoxycarotenoid (*NCED*) genes involved in abscisic acid (ABA) biosynthesis. Figures **a** through **o** represent the expression level of genes in vegetative and reproductive organs under ideal growing conditions (22°C) or in response to severe temperature stress (cold = 0°C, heat = 37°C). For a given gene (*NCED2, NCED3, NCED5, NCED6, NCED9*) and tissue (cauline leaves, inflorescence meristem, developing silique) astericks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to plants grown at 22°C. Data correspond to mean and standard deviation (SD) of three biological replicates. RT-qPCR data were normalized to the expression of the internal reference gene PP2A (*At1g13320*). Significant developmental patterns (22°C) of gene expression and associated P-values are discussed in text. P-values determined according to a two-tailed studentized t-test (equal variance). Note relative expression scale for *NCED2* and *NCED5* in cauline leaves is higher than for inflorescence meristems and developing siliques.

upon the tissue sampled (Fig. 5.4d-f) (Nambara and Marion-Poll 2005; Barrero et al. 2006). In cauline leaves, cold and heat stress repressed *NCED3* transcripts (Fig. 5.4d) but within inflorescence meristems and developing siliques, cold and heat stress activated *NCED3* expression (Fig. 5.4e,f).

Across tissues isolated from plants grown at 22°C certain family members (*NCED3, NCED6*) were expressed at high levels in cauline leaves relative to inflorescence meristems or developing siliques. For example, *NCED3* expression was 23.4-fold ($P < 0.01$) higher in cauline leaves than inflorescence meristems (Fig. 5.4d,e). Other family members had relative expression levels highest in developing siliques (*NCED9*) or inflorescence meristems (*NCED2, NCED5*) (Fig. 5.4).

5.4.4 Expression analysis of 8'-hydroxylase genes (*CYP707A1-A4*) involved in ABA catabolism

In Arabidopsis a major ABA catabolic pathway is mediated by four members of the CYP707A family of cytochrome P450 monooxygenases which encode enzymes (ABA hydroxylases) capable of converting abscisic acid to 8'-hydroxy-ABA or 9'-hydroxy-ABA, which are subsequently isomerized to form phaseic acid or dihydrophaseic acid (Fig. 5.1) (Kushiro et al. 2004; Okamoto et al. 2011). In cauline leaves, expression of all four *CYP707A* genes was strongly activated by cold stress, whereas heat stress resulted in smaller albeit significant increases in the expression of specific family members (*CYP707A1, CYP707A4*) (Fig. 5.5). Cold stress also coordinately activated *CYP707A* genes in inflorescence meristems, although to a lesser extent than observed in cauline leaves (Fig. 5.5). Within cauline leaves exposed to cold stress *CYP707A1-A4* transcripts increased 5.3 to 33.4-fold whereas for inflorescence

meristems exposed to cold stress *CYP707A1-A4* transcripts increased 1.8 to 8.9-fold (Fig. 5.5). Where cold stress resulted in widespread and coordinated activation of ABA catabolism, heat stress triggered a more subtle activation (*CYP707A1, CYP707A4*) or even repression (*CYP707A2*, *CYP707A3*) of specific gene family members (Fig. 5.5). *CYP707A1* and *CYP707A3* genes were highly expressed in inflorescence meristems relative to cauline leaves and developing siliques. For example, *CYP707A1* expression was 8.2-fold higher ($P < 0.05$) in inflorescence meristem than cauline leaves (Fig. 5.5a,b). However, for *CYP707A2* and *CYP707A4*, relative gene expression levels were lowest in inflorescence meristems amongst all tissues analyzed (Fig. 5.5).

5.4.5 Expression analysis of genes involved in ABA transport and homeostasis

In cauline leaves, cold and heat stress had opposing effects on the relative expression level of genes involved in ABA transport (*ABCG25, ABCG40*) and glucosylation (*UGT71B6*) (Fig. 5.6a,d,g). All three genes were activated by cold stress in cauline leaves, but repressed by heat stress (Fig. 5.6). Within inflorescence meristems both cold and heat stress treatments coordinately down-regulated the expression of *ABCG25, ABCG40* and *UGT71B6* (Fig. 5.6b,e,h). In developing siliques, these genes were activated by cold stress in a similar manner to that observed in cauline leaves, but were unaffected by heat stress (Fig. 5.6c,f,i). *AtBG1* was also differentially expressed in response to temperature stress (Fig. 5.6). Within cauline leaves both cold and heat stress activated *AtBG1* expression (Fig. 5.6j). However, within inflorescence meristems similar temperature treatments down-regulated *AtBG1* transcripts (Fig. 5.6b).

In the absence of stress, *ABCG25* and *ABCG40* expression was highest in inflorescence meristems relative to cauline leaves and developing siliques (Fig. 5.6a-f).

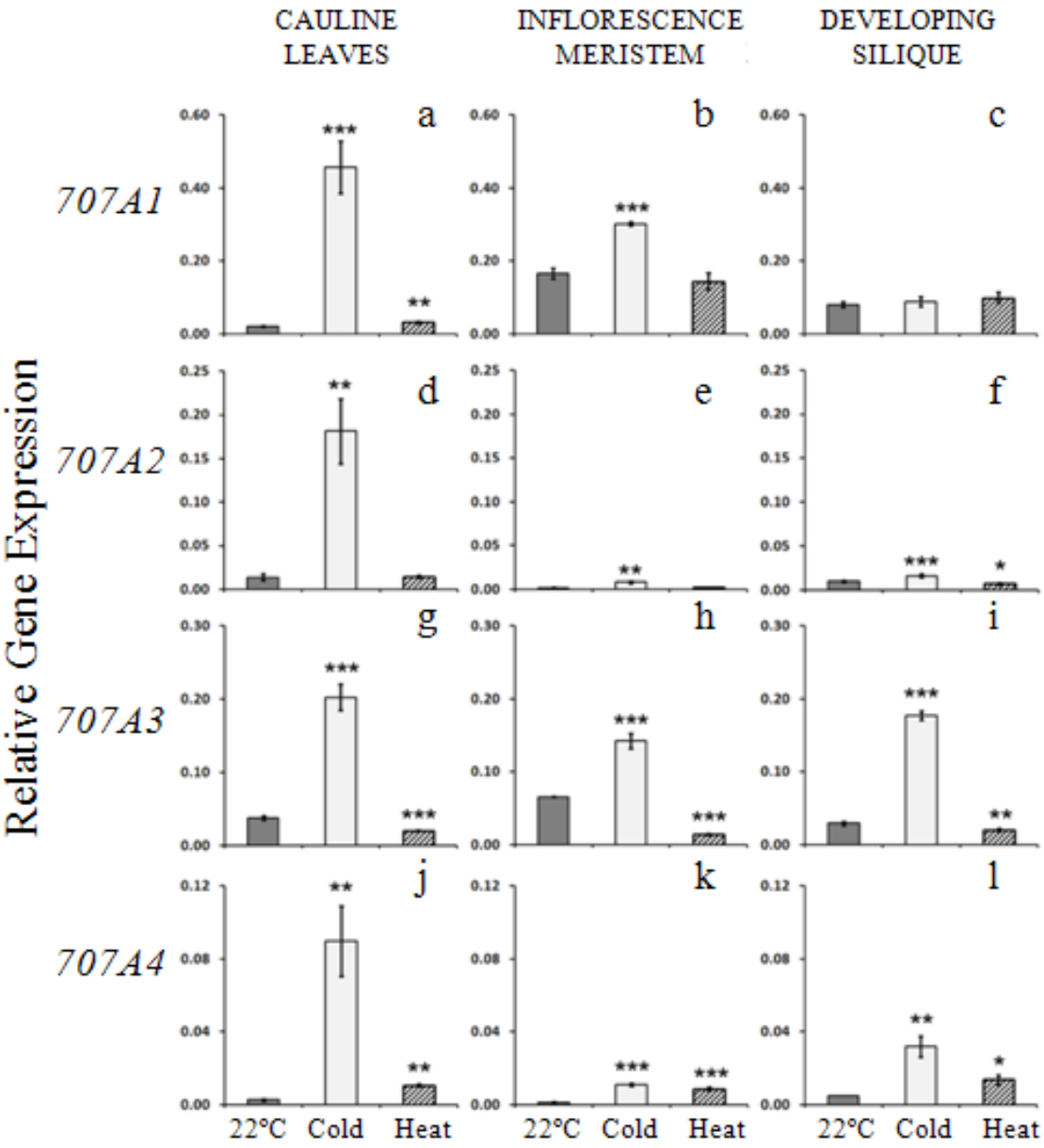

Fig. 5.5 Expression of genes involved in abscisic acid (ABA) catabolism. Figures **a** through **l** represent the expression level of catabolic genes in vegetative and reproductive organs under ideal growing conditions (22°C) or in response to severe temperature stress (cold = 0°C, heat = 37°C). For a given gene (*CYP707A1-A4*) and tissue (cauline leaves, inflorescence meristem, developing silique) astericks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to plants grown at 22°C. Data correspond to mean and standard deviation (SD) of three biological replicates. RT-qPCR data were normalized to the expression of the internal reference gene PP2A (*At1g13320*). Significant differences in gene expression (developmental) amongst tissues and associated P-values are discussed in text. P-values were determined according to a two-tailed studentized t-test (equal variance).

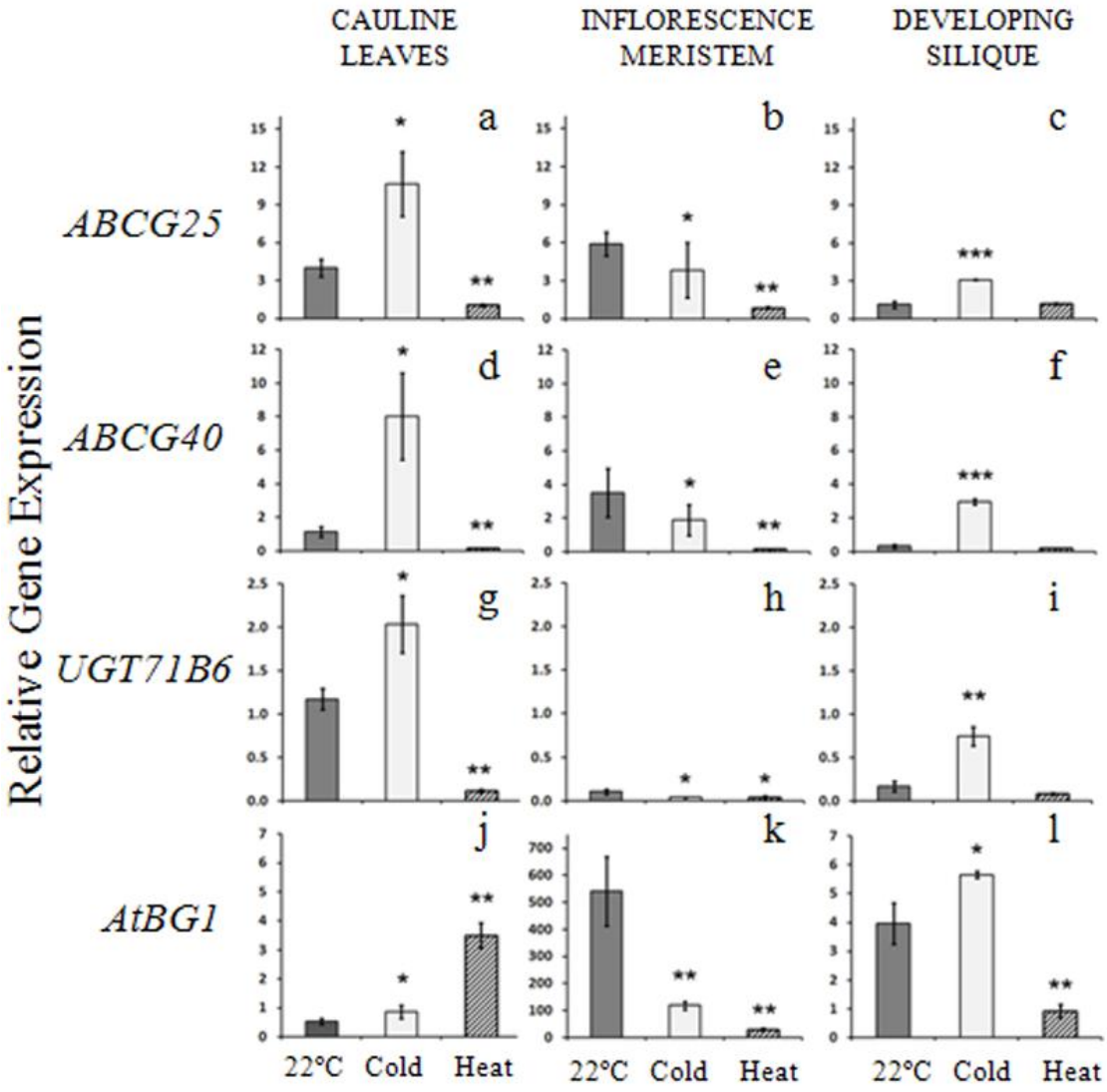

Fig. 5.6 Expression of genes involved in transport, conjugation, or hydrolysis of abscisic acid (ABA) or abscisic acid-glucose ester (ABA-GE). Figures **a** through **l** represent the expression level of key genes (*ABCG25, ABCG40, UGT71B6, AtBG1*) in vegetative and reproductive organs under ideal growing conditions (22°C) or in response to severe temperature stress (cold = 0°C, heat = 37°C). For a given gene (*ABCG25, ABCG40, UGT71B6, AtBG1*) and tissue astericks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate significantly altered gene expression relative to plants grown at 22°C. Data correspond to mean and standard deviation (SD) of three biological replicates. RT-qPCR data were normalized to the expression of the internal reference gene *PP2A* (*At1g13320*). Significant differences in gene expression (developmental) and associated P-values are discussed in text. P-values were determined according to a two-tailed studentized t-test (equal variance). Note change in scale of relative gene expression for *AtBG1* across reproductive tissues.

For example, *ABCG40* expression was 3.1-fold ($P < 0.05$) higher in inflorescence meristems relative to cauline leaves (Fig. 5.6d,e). For *UGT71B6*, the highest relative levels of expression were observed in cauline leaves (Fig. 5.6g), being 10.9-fold higher ($P < 0.001$) than in inflorescence meristems (Fig. 5.6g,h). From a developmental perspective, *AtBG1* expression levels were exceptionally high in inflorescence meristems (note adjusted scale) (Fig. 5.6). When compared to cauline leaves, *AtBG1* expression levels were 1018-fold ($P < 0.05$) and 7.5-fold ($P < 0.05$) higher in inflorescence meristems and developing siliques, respectively (Fig. 5.6j-l).

5.4.6 ABA content of vegetative and reproductive organs following cold and heat stress

Fig. 5.7 displays the ABA content of cauline leaves, inflorescence meristems and developing siliques of Arabidopsis plants under control conditions or following exposure to cold and heat stress. In cauline leaves cold and heat stress caused ABA levels to increase 2.5- and 2.1-fold, respectively, relative to controls (Fig. 5.7a). Application of cold stress led to a significant increase in ABA content of inflorescence meristems, with ABA levels rising 5.5-fold relative to non-stressed controls (Fig. 5.7b). Under our experimental conditions the ABA content of inflorescence meristems exposed to heat stress did not differ significantly from plants grown under control conditions (Fig. 5.7b). In contrast to significant changes in ABA content of cauline leaves or inflorescence meristems following cold or heat stress, no significant differences in ABA content of developing siliques were detected following exposure to cold or heat stress (Fig. 5.7c).

The ABA content of inflorescence meristems was highest amongst all vegetative and reproductive organs (non-stressed) examined, decreasing along the order

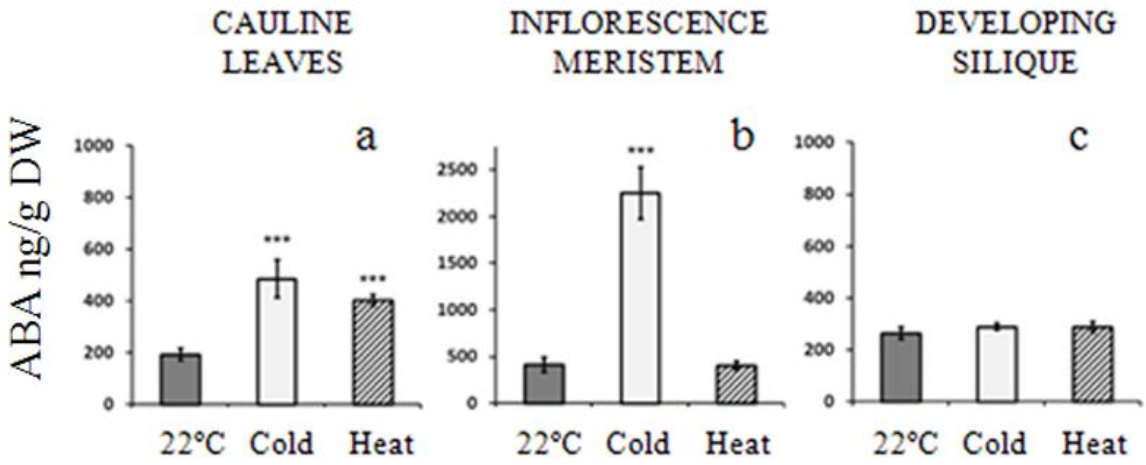

Fig. 5.7 Measurement of ABA levels in vegetative and reproductive organs (cauline leaves, inflorescence meristem, developing silique) of *Arabidopsis thaliana* plants exposed to severe temperature stress (cold = 0°C, heat = 37°C). For a given tissue, astericks ($P \leq 0.05$ = *, $P \leq 0.01$ = **, $P \leq 0.001$ = ***) indicate a significant difference in ABA content relative to plants grown at 22°C. Error bars represent standard errors (SE).

inflorescence meristem > developing silique > cauline leaves. The ABA content of inflorescence meristems was 2.1-fold higher ($P < 0.001$) than in cauline leaves.

5.5 Discussion

In plants, cellular ABA levels and ABA homeostasis are regulated through the coordinated action of numerous genes involved in ABA biosynthesis, catabolism and transport, alongside loci mediating the conjugation or hydrolysis of ABA and ABA-GE (Nambara and Marion-Poll 2005; Lee et al. 2006; Priest et al. 2006; Umezawa et al. 2010). In the current study, we examined how vegetative and reproductive organs of *Arabidopsis thaliana* differ with respect to transcriptional regulation of ABA metabolism

and homeostasis in response to abiotic stress. To our knowledge this is the first quantitative study to assess characterized genes involved in ABA biosynthesis and catabolism alongside functional genes (*ABCG25*,*ABCG40*, *UGT71B6*, *AtBG1*) mediating ABA transport and homeostasis. By comparing the transcriptional response of vegetative and reproductive organs exposed to identical levels of abiotic stress, we identified several novel features of ABA homeostasis that operate at the whole plant level.

5.5.1 Cold and heat stress differentially regulate ABA biosynthesis and catabolism

Our results indicate cold and heat stress lead to widespread activation of ABA biosynthesis in vegetative and reproductive organs (Fig. 5.3; Fig. 5.4). However, depending upon the organ, cold and heat stress appear to differentially activate select ABA biosynthesis genes over others. For a number of ABA biosynthesis genes (*ABA1*, *ABA2*, *ABA4*, *AAO3*, *NCED3*, *NCED5*, *NCED6*) temperature stress simultaneously activated or down-regulated gene expression in an organ-specific manner. Previous functional studies of *NCED* and *CYP707A* gene families revealed individual members display complex developmental patterns of expression and subcellular targeting (Tan et al. 2003; Saito et al. 2004; Lefebvre et al. 2006; Okamoto et al. 2009). In the current study, high levels of *NCED3* and *NCED9* expression observed in cauline leaves and developing siliques were in agreement with previous studies (Fig. 5.4) (Tan et al. 2003). However, down-regulation of *NCED3* transcripts in cauline leaves following cold and heat stress was unexpected (Fig. 5.4). A distinguishing feature of the current study is the duration of stresses (24 h) applied to vegetative and reproductive organs. At this juncture certain tissues may have transitioned from ABA biosynthesis to ABA catabolism (Ji et al. 2011). *NCED3* expression is often considered rate-limiting for ABA biosynthesis in

response to abiotic stress (Barrero et al. 2006). However, this gene is also regulated by sugars and chromatin modifications, suggesting regulatory control of *NCED3* may be more complex than initially thought (Huang et al. 2008; Ding et al. 2011). Observations in this and other studies indicate ABA metabolism is highly coordinated during reproductive development (Tan et al. 2003; Saito et al. 2004; Nitsch et al. 2009). Isolating tissue-specific *cis*-or-*trans* acting factors capable of activating or repressing ABA biosynthesis gene expression may represent one approach to elucidate the regulatory control of ABA metabolism during plant development.

Previous studies of *CYP707A* genes determined family-wide induction occurs in response to cold stress or exogenous ABA (Saito et al. 2004; Zhou et al. 2007). However, in certain physiological contexts (i.e., high temperature or elevated glucose levels), limited induction of *CYP707A* genes emerged as a mechanism to elevate endogenous ABA levels (Toh et al. 2008; Zhu et al. 2009). Our results indicate cold stress broadly activates ABA catabolism within vegetative and reproductive organs (Fig. 5.5). However, limited induction of *CYP707A* genes occurs following heat stress (Fig. 5.5). These observations indicate cold and heat stress differentially target genes responsible for ABA catabolism. In the current study, limited induction (or even repression) of *CYP707A* gene expression may contribute to the increased ABA content of cauline leaves exposed to heat stress (Fig. 5.7). Collectively, these observations indicate comparable increases in the ABA content of vegetative tissues following cold and heat stress (Fig. 5.7) may be supported by vastly different transcriptional profiles regulating ABA metabolism and turnover, in addition to accumulation of ABA-related catabolites.

5.5.2 Regulation of ABA homeostasis and transport

As observed for ABA biosynthesis and catabolism, genes involved in ABA homeostasis and transport responded to abiotic stress in an organ-specific manner. Across all organs ABA transporter (*ABCG25, ABCG40*) and *UGT71B6* expression appeared coordinated, suggesting pathways responsible for ABA transport and formation of ABA-GE are coupled to some degree. *AtBG1* also responded to cold and heat stress in an organ-specific manner. However, across organs the response of *AtBG1* to cold and heat stress was unique compared to ABA transporters and *UGT71B6*. Within inflorescence meristems exposed to cold and heat stress, all four genes involved in ABA transport and homeostasis were repressed (Fig. 5.6) while genes involved in ABA biosynthesis (*ABA1, ABA2, ABA4, AAO3*) were upregulated (Fig. 5.3). In these meristems, transcriptional regulation of ABA biosynthesis appeared independent from pathways mediating ABA transport and homeostasis. However, cauline leaves were found to have certain ABA biosynthesis genes (*NCED2, NCED5, NCED6*) differentially regulated by cold and heat stress in exactly the same manner as ABA transporters (*ABCG25, ABCG40*) and *UGT71B6* (Fig. 5.4; Fig. 5.6). In this organ, transcriptional regulation of ABA metabolism and homeostasis appeared coordinated.

A major finding from the current study is that during development and in response to abiotic stress, plant organs in close physical proximity can undergo substantially different transcriptional programs regulating ABA homeostasis. At present, very little molecular information is available to characterize how ABA is mobilized and transported during systemic ABA responses. Previous physiological studies utilizing radiolabelled ABA determined mature leaves possess the greatest capacity to produce

ABA (Zeevaart and Creelman 1988). In turn, the efficient translocation of ABA and photoassimilates from mature leaves (source) to immature leaves, buds and reproductive structures (sinks) causes the latter organs to accumulate high levels of ABA (Zeevaart and Creelman 1988). Long-distance transport of ABA or ABA conjugates also participates in the transmission of hydraulic or chemical signals influencing stomatal conductance in response to water deficit (Sauter et al. 2002; Schachtman and Goodger 2008). In the current study, we identified several molecular features of ABA metabolism and homeostasis that contribute towards the mobilization of ABA during development and in response to abiotic stress. For example, in the absence of stress, inflorescence meristems are characterized by exceptionally high expression of *AtBG1*, an intracellular β-glucosidase involved in hydrolysis of ABA-GE (Fig. 5.6) (Schroeder and Nambara 2006). Transporters responsible for import (*ABCG40*) and export (*ABCG25*) of ABA are also highly expressed in inflorescence meristems (Fig. 5.6). Previous functional studies determined ABA generated by ER-localized AtBG1 is secreted into the apoplastic space of plant cells, with AtBG1-mediated increases in ABA coupled with cytosolic pathways regulating *de novo* ABA biosynthesis (Lee et al. 2006). Our results suggest inflorescence meristems are capable of generating substantial quantities of ABA, a considerable portion of which may be secreted into the apoplastic space in the absence of stress. In addition to AtBG1, an as yet unidentified stress-inducible extracellular β-glucosidase may also influence extracellular ABA levels in Arabidopsis (Dietz et al. 2000). Although interactions between sugar and ABA signaling have been known for some time, an intriguing area of future study surrounds the role of intracellular or extracellular ABA levels in driving the expression, localization or activity of invertases, invertase inhibitor

proteins and sucrose transporters involved in carbohydrate metabolism and transport (Oliver et al. 2007; Hoth et al. 2010; Ruan et al. 2010).

Previous physiological studies revealed cold and heat stress, in addition to drought stress, simultaneously elevate ABA and ABA-GE levels in leaves (Daie and Campbell 1981; Zeevaart 1983). However, with drought stress or repeated stress-recovery cycles, ABA-GE levels may even exceed those of free ABA (Sauter et al. 2002; Zeevaart 1983). ABA and ABA-GE also exhibit subtle differences in cellular localization that may impact stability or access to enzymes involved in ABA metabolism (Bray and Zeevaart 1985; Lehmann and Glund 1986).

5.5.3 ABA functions in vegetative and reproductive development

Amongst reproductive organs analyzed in the current study only inflorescence meristems exposed to cold stress displayed significant increases in ABA content (Fig. 5.7b). In cauline leaves, cold and heat stress treatments caused similar increases in ABA levels (Fig. 5.7b). Currently, the role for ABA in reproductive development and abiotic stress tolerance of reproductive structures is ambiguous. Recent studies propose excessive ABA accumulation negatively impacts the abiotic stress tolerance of reproductive tissues (Oliver et al. 2007; Ji et al. 2011). However, other studies found no such correlations (Saini et al. 1982; Dembinska et al. 1992). Nonetheless, in the absence of stress, ABA appears to accumulate within meristematic tissues of roots and shoots (Schraut et al. 2004; Peng et al. 2006; Priest et al. 2006; Nitsch et al. 2009; Wong et al. 2009). In the current study, differences in ABA accumulation, metabolism or signaling between inflorescence meristems exposed to cold or heat stress may affect meristem dormancy and the length of developmental phases contributing to flowering time and

fruit development. For example, at the reproductive stage of development cold and heat stress exert opposing roles in delaying or accelerating floral induction and flowering time, respectively (Balasubramanian et al. 2006; Hasdai et al. 2006). However, in other physiological contexts, cold and heat stress trigger similar perturbations to processes affecting photosynthesis and assimilation of carbon (Barnabás et al. 2008; Thakur et al. 2010).

In summary, the current study offers new insight into the transcriptional regulation of ABA metabolism and transport at the whole plant level. This work reveals vegetative and reproductive organs differ substantially with respect to transcriptional regulation of ABA homeostasis during development and in response to abiotic stress. However, it is important to note several genes (*NCED3, AAO3, AtBG1*) analyzed in the current study are subject to additional levels of regulatory control (e.g. epigenetic, post-transcriptional, post-translational) that modify the abundance, localization or activity of enzymes involved in ABA metabolism. Low and high temperature extremes assessed in the current study also dramatically alter pathways responsible for RNAi-mediated gene silencing and genesis of small RNAs (Szittya et al. 2003; Yu et al. 2012). Abiotic stress treatments and even metabolic status (rapidly growing or inactive) of tissues also influence mRNA turnover and translation in plants (Volkov et al. 2003; Narsai et al. 2007; Matsuura et al. 2010). In the future, use of microtracer techniques and radiolabelled ABA, along with available Arabidopsis mutants, may serve as valuable tools in elucidating the molecular control of ABA metabolism and intercellular ABA transport at the whole plant level.

Table 5.1S List of oligonucleotide sequences utilized for RT-qPCR analysis of genes involved in ABA metabolism, transport and homeostasis.

Gene	AGI Code		Oligo Sequence
ABA1	*At5g67030*	Forward	5'-TGGGTGCAGATGGCATTTGGTCTA-3’
		Reverse	5'-CCACCACCAACATCCGAAGAAACA-3’
ABA2	*At1g52340*	Forward	5'-CTCGCTTTGGCTCATTTGC-3'
		Reverse	5'-CCGTCAGTTCCACCCCTTT-3'
ABA4	*At1g67080*	Forward	5'-AATGACTCTTGCTTCTGCTTGGAT-3'
		Reverse	5'-GCTTTGGTTACGAAATGCGAAACGAT-3'
AAO3	*At2g27150*	Forward	5'-TGGAAGTGGACCTTGTGACAGGAA-3’
		Reverse	5'-AACCCGATGCCTTGAACAAATGCTCC-3’
NCED2	*At4g18350*	Forward	5’-GCGTGCATTAATCTCACACGAGCA-3’
		Reverse	5’-ATGCAGTCAGGGATTGTTCCTTCG-3’
NCED3	*At3g14440*	Forward	5’-GGAATCCGGTGAACTCTTCGCTTT-3’
		Reverse	5’-AAACGACTTGCTGGTCAGGTACGA-3’
NCED5	*At1g30100*	Forward	5’-TTCGCCGTCATCCTCCGTTAGTTT-3’
		Reverse	5’- AGGGTTCCAACGGGAAGTGTCTTT-3’
NCED6	*At3g24200*	Forward	5’-GGCTACGATGCTCGACAAGATTGA-3’
		Reverse	5’-AACCGGACATTCATTAACCGGAGC-3'
NCED9	*At1g78390*	Forward	5’-TCTCCGACATTCAAACCACCGTCT-3’
		Reverse	5’-GCTCGTGTGAGATCATGGCGTTT-3
CYP707A1	*At4g19230*	Forward	5'-TTGTTTCTCACTCTCTTCGCCGGA-3'
		Reverse	5'-AAGTTTCTCCGACGTAAGGCCAAC-3'
CYP707A2	*At2g29090*	Forward	5-TCCTCCAAACCCTTTCCTCTTGGA-3'
		Reverse	5'-CTTTGAAAGAAGTGAGGCCCGCAA-3'
CYP707A3	*At5g45340*	Forward	5'-GGCGGCTCTGTTTCTCTGTTTACT-3'
		Reverse	5'-TTGGAATGTTTCGCCGACGTAAGG-3'
CYP707A4	*At3g19270*	Forward	5’-TCGAGCACATTGCCCTTTCTTCCT-3'
		Reverse	5’-CCACATCAAAGGCGAACTGCAAGA-3'
BCG25	*At1g71960*	Forward	5’-CGCCATGGCTTACTTTGAATCCGT-3’
		Reverse	5’-TGTATCATAAGCCGTGACCAGCGT-3’
BCG40	*At1g15520*	Forward	5’-ATGTTCTGGGACCTTGGAGGCAAA-3’
		Reverse	5’- AGCATAAGGCATGGCGGAGTACAT-3’
AtBG1	*At1g52400*	Forward	5’-ACAAGGCGAGGTTCGGACTTTACT-3’
		Reverse	5’-AGTTCTTCCCTCAGCTTGGAGGTT-3’
UGT71B6	*At3g21780*	Forward	5’-CAACCGCCTCCGGTACGAAATAATC-3’
		Reverse	5’-AGGCGCGTCTGGTAGAGTGGAAT-3’
PDF subunit PP2A	*At1g13320*	Forward	5'-TAACGTGGCCAAAATGATGC-3'
		Reverse	5'-GTTCTCCACAACCGCTTGGT-3'
PDF subunit PP2A	*At1g13320*	Forward	5’-CATGTTCCAAACTCTTACCTGCGG-3’
		Reverse	5’-TGGGTCTTCACTTAGCTCCACCAA-3’

6.0 GENERAL DISCUSSION AND CONCLUSIONS

In recent years considerable progress has been made towards clarifying molecular and cellular components of the ABA signal transduction pathway in plants. However, many aspects of ABA metabolism, signaling and physiology remain to be elucidated. The present study was undertaken with the objective of functionally characterizing ***GE****M-****R****ELATED* ***5*** (*GER5*), a member of the ABA-responsive GRAM domain gene family in *Arabidopsis thaliana*. The findings presented in chapter 1 indicate that *GER5*/At5g13200 is broadly expressed in tissues of *Arabidopsis thaliana*, and plays a critical role in the early reproductive development of plants. In this work, a full-length version of *GER5* was isolated and utilized to generate transgenic lines which overexpress ($GER5^{OE}$) or silence ($GER5^{RNAi}$) *GER5*. A loss-of-function T-DNA insertion line was also obtained from GABI-KAT, genotyped and named *ger5-2*. Mutant and transgenic lines associated with loss- or gain-of-*GER5* function display numerous defects in reproductive development affecting inflorescence architecture, floral abortion, silique morphology and patterning, in addition to abortion and senescence of developing seeds. The variable penetrance of reproductive phenotypes in mutant and transgenic lines also led to a broader examination of two closely related GRAM genes, ***GE****M-****R****ELATED* ***1*** (*GER1*/At1g28200) and ***G****LABRA2* ***E****XPRESSION* ***M****ODULATOR* (*GEM*/At2g22475) in reproductive organs. RNA *in situ* studies revealed *GER5*, *GER1* and *GEM* had both unique and overlapping expression domains in reproductive organs. The expression domain of *GER5* closely overlapped with sites of ABA accumulation in reproductive organs. Gene expression studies revealed all three GRAM genes were broadly expressed during plant development and were responsive to exogenous ABA or cold stress.

However, in mutant backgrounds deficient in ABA biosynthesis or sugar signaling, these same genes were differentially expressed in a tissue-specific manner. Mature seeds of *ger5-2* mutants also exhibited reduced sensitivity to ABA during seed germination assays. During seed development microarray analysis of aborting and developing seeds isolated from *ger5-2* mutants revealed underlying transcriptional changes in carbohydrate metabolism, hormone signaling and catabolic processes. Collectively, this study determined *GER5* and closely related members of the ABA-responsive GRAM gene family function during early stages of reproductive development and also modulate the response of tissues to ABA during seed dormancy and germination.

The second study in this thesis focused on the environmental and hormonal regulation of autophagy relative to ABA biosynthesis and signaling in autotrophic and heterotrophic plant organs. Utilizing abiotic stress treatments which trigger ABA biosynthesis, in addition to publicly available mutants deficient in ABA biosynthesis or sugar signaling, the transcriptional response of *ATG* and ABA response genes was assessed. In response to cold stress a number of *ATG* genes were upregulated in both source and sink organs. Members of an ABA-responsive gene family (*AtHVA22*) known to negatively regulate programmed cell death and autophagy were expressed at exceptionally high levels across both source and sink organs exposed to cold stress. Other characterized negative regulators of autophagy (*AtTOR*) involved in perceiving nutrient status were down-regulated specifically within inflorescence meristems exposed to cold or heat stress. In mutant backgrounds deficient in ABA biosynthesis and sugar signaling many *ATG* and ABA response genes were differentially regulated. In the current study it was difficult to discern a clear relationship between ABA biosynthesis

and the regulation *ATG* genes. In several instances the transcriptional response of *ATG* or ABA response genes to abiotic stress or defects in sugar signaling differed depending upon the tissue analyzed. At a morphological level monodansylcadaverine (MDC) staining was utilized to detect autophagosomes or autophagic bodies in tissues exposed to abiotic stress or nutrient deprivation. For carbon-starved Arabidopsis seedlings, both cold and heat stress treatments increased MDC staining of root segments. In root cells exposed to these same temperature extremes, motile autophagosomes (cytoplasm) coalesced into structures resembling autophagic bodies (vacuoles). MDC staining experiments further revealed that specific cell types of vegetative and reproductive organs were characterized by elevated levels of autophagic activity. Within cauline leaves exposed to carbon starvation (darkness), MDC preferentially labeled autophagosomes in stomatal guard cells and subsidiary cells. During seed development and germination, seed coat cells (integuments) and aleurone cells also stained prominently for autophagosomes or autophagic bodies.

The final study in this thesis evaluated whether rapidly growing reproductive organs (e.g. inflorescence meristems, developing siliques) of *Arabidopsis thaliana* could be characterized by unique features of ABA metabolism and homeostasis relative to subtending cauline leaves. In addition, whether the transcriptional response of reproductive organs following abiotic stress differs from the response of vegetative organs exposed to identical levels of stress. Across vegetative and reproductive organs, cold and heat stress treatments induced ABA biosynthesis genes to similar extents, although specific genes were clearly more responsive to cold stress (*ABA1*, *NCED2)* or heat stress (*ABA2*, *ABA4*). For *CYP707A* genes which function in ABA catabolism, cold

stress triggered a strong, coordinated upregulation of all family members across tissues assessed. However, this same pattern of gene expression did not emerge for plants exposed to heat stress. Collectively, for vegetative organs exposed to cold and heat stress, comparable increases in the ABA content of cauline leaves were supported by vastly different transcriptional programs regulating ABA metabolism and homeostasis. Depending upon organs analyzed recently discovered genes involved in the hydrolysis, conjugation and transport of ABA were similarly or differentially expressed in response to cold and heat stress. In a developmental context a number of key ABA biosynthesis genes (*ABA1*, *ABA2*) and a novel gene (*AtBG1*) mediating the hydrolysis of ABA-GE to ABA were expressed at extremely high levels in developing inflorescences. Taken together the individual studies contained within this thesis have not only provided new information regarding the role of ABA-responsive GRAM genes during plant development and stress responses, but have also demonstrated reproductive organs may be characterized by novel features of ABA metabolism, transport, homeostasis and signaling that differ significantly from tissues associated with classical ABA responses (e.g. seed dormancy, stomatal closure), where the majority of ABA-response loci have been isolated and characterized.

Based on family-wide assessments of gene expression and the identification of myotubularin homologs (*AtMTM1*, *AtMTM2*) in *Arabidopsis thaliana*, future characterization efforts of the GRAM and myotubularin (MTM) gene families may seek to determine the functional significance of GRAM and C2 domains in membrane targeting and subcellular localization. In addition, at a biochemical level, whether GRAM and C2 domains encoded by these gene families function as bona fide

phosphoinositide or Ca^{2+} binding motifs, both *in vitro* and *in planta*. Protein-protein interaction studies may also identify family members which are capable of forming functional homo- or hetero-dimer complexes, or potentially compete for common targets.

Relative to ABA signaling, several PI species which serve as substrates of GRAM domain proteins in yeast and humans also mediate the formation of reactive oxygen species (ROS) involved in ABA-mediated stomatal closure (Jung et al. 2002; Park et al. 2003). Upon resolving the PI and Ca^{2+}-binding properties of GRAM and MTM proteins in plants, future efforts may be directed towards manipulating PI metabolism using inhibitors (e.g. wortmannin, LY294002) or genetic approaches, and examining how such perturbations modify the localization or activity of GRAM proteins. Takemiya et al. (2012) recently reported Arabidopsis PRSL1 (PP1 regulatory subunit2-like protein1), a GRAM domain protein, localizes to the cytoplasm of guard cells and functions as a regulatory subunit of protein phosphatase 1 (PP1), the latter serving as a point of cross-talk between blue light and ABA signaling pathways. With regards to ABA dependent sugar signaling pathways, a cytosolic invertase (CINV1) and SnRK1 also interact with enzymes (PIP5K9, 5PTase13) involved in PI metabolism (Lou et al. 2007; Ananieva et al. 2008). These respective interactions were reported to negatively regulate invertase activity or modulate SnRK1 activity in a nutrient-dependent manner. A model depicting how GRAM and myotubularin genes may participate in ABA signaling during plant development and stress responses in shown in Fig. 6.1.

Beyond cellular and molecular function(s) of GRAM and MTM genes in ABA, abiotic stress and phospholipid signaling, reproductive phenotypes observed in the present study and others (Chen et al. 2009; Fitzpatrick et al. 2011) raised intriguing

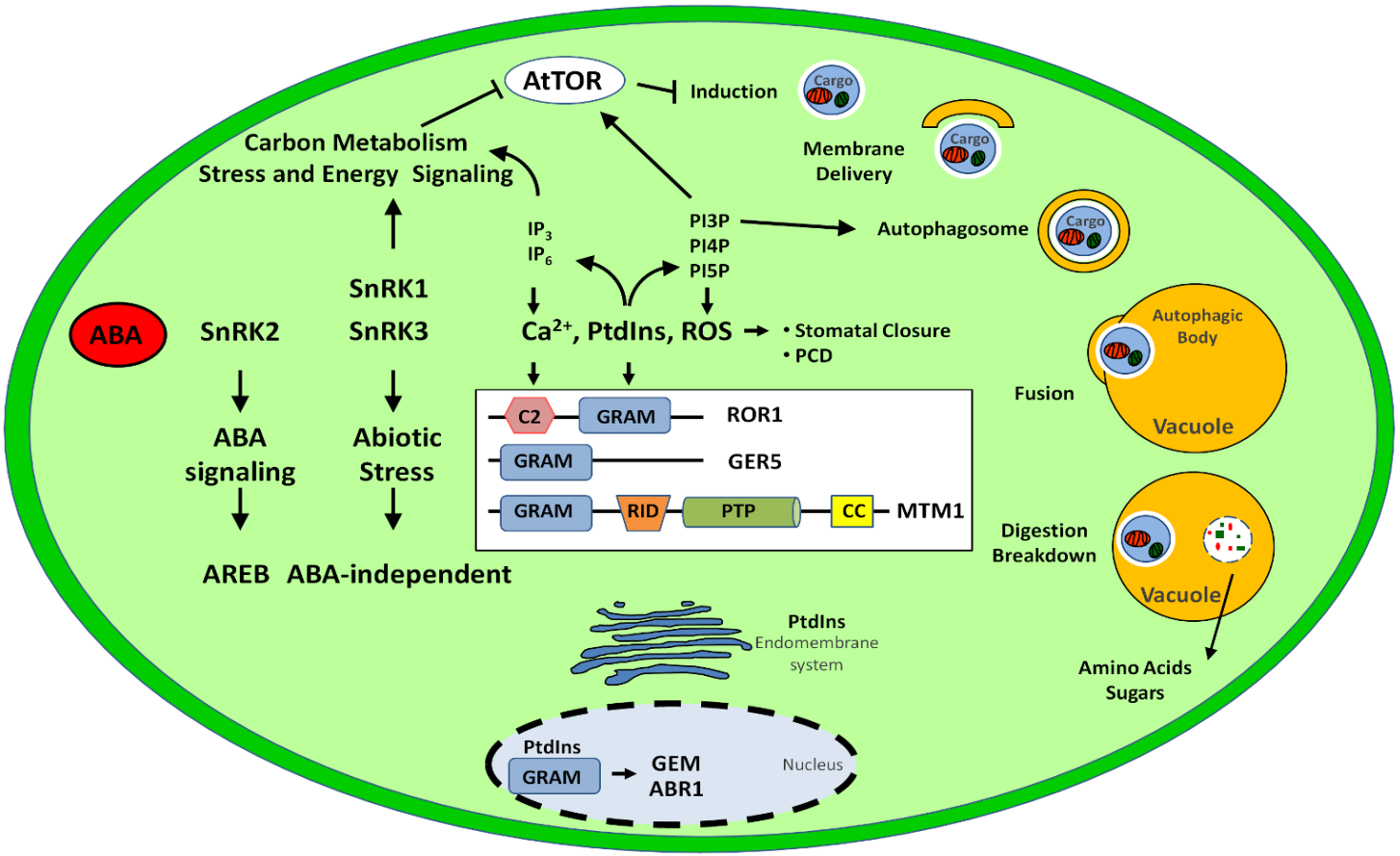

Fig. 6.1 Working model summarizing role of GRAM proteins in plant growth, development and stress response. With the exception of *AtMTM1* and *AtMTM2*, genes encoding GRAM domain proteins lack catalytic domains required to dephosphorylate lipids or proteins *in vivo*. However, non-catalytic GRAM proteins may form protein-protein interactions with catalytic serine/threonine phosphatases (e.g. PP1). In yeast and humans, GRAM proteins participate in synthesis and degradation of phosphinositide species which initiate

Figure 6.1 (continued) autophagy, a conserved eukaryotic process for degrading cytoplasmic components or superflous organelles during development or in response to carbon starvation. ABA appears to function as a plant specific regulator of autophagy and GRAM genes are transcriptionally responsive to ABA, nutrient deprivation or environmental stress. Phosphoinositide species (PI3P, PI4P) bound by characterized eukaryotic GRAM domain proteins also function as secondary messengers in the ABA signal transduction pathway leading to ROS formation, stomatal closure and programmed cell death. In the ABA signaling pathway related phosphoinositide species (IP_3, IP_6) also function as secondary messengers to modulate sugar signaling (e.g. SnRK1) and intracellular Ca^{2+} dynamics, which may modify the localization or activity of GRAM proteins which contain C2 domains. In all eukaryotes the multimeric SnRK1 (subfamily 1) and TOR protein kinases interact in opposing manners to regulate nutrient and energy driven processes such as ATP production, protein translation and autophagy. In plants, the SnRK family (SnRK1, 2 members) has expanded and diverged to include plant specific SnRK2 (10 members) and SnRK3 (25 members) sub-families. SnRK2 and SnRK3 play central roles in ABA signaling in addition to the response of plant cells to nutrient deprivation, drought, cold, salt and osmotic stress. C2 = Ca^{2+}/phospholipid domain, RID = Rac-induced recruitment domain, CC = coiled coil domain

question regarding the significance of ABA metabolism, signaling and autophagy at this stage of the plant lifecycle. For example, in response to acute salt stress, ovule abortion and senescence in Arabidopsis is accompanied by changes in mitochondrial membrane potential, accumulation of ROS, in addition to ultrastructural changes resembling autophagy (Hauser et al. 2006). Under long day conditions which accelerate spike and floret development in wheat, Ghiglione et al. (2008) proposed that a developmentally generated sugar starvation program initiated autophagy and programmed cell death to decrease the number of fertile florets at anthesis. Moving forward, a key question analogous to that encountered in basal plant immunity is whether autophagy fulfils a ‘pro-death’ or ‘pro-survival’ role during programmed cell death events associated with senescence or reductions in plant fecundity (Ghiglione et al. 2008; Lenz et al. 2011). From loss-and gain-of-function studies conducted to date, GRAM domain proteins

(VAD1, CaABR1) appear to function as both positive and negative regulators of cell death during plant-pathogen interactions (Bouchez et al. 2007; Choi and Hwang 2011).

Our transcriptional analysis of key genes involved in ABA metabolism, transport and homeostasis further revealed that during development and in response to abiotic stress reproductive organs are characterized by novel aspects of ABA metabolism and physiology. In economically important crop species (e.g. wheat, maize) the excessive accumulation of ABA and sugars within reproductive tissues has been negatively correlated with the stress tolerance of reproductive organs (Ji et al. 2011; Setter et al. 2011). From these same studies, stress-tolerant germplasm appears to be characterized by modest increases in ABA levels and maintenance of growth-related processes as opposed to elevated expression of genes associated with stress-response pathways (Ji et al. 2011; Setter et al. 2011). Therefore, in attempting to improve the abiotic stress tolerance of diverse germplasm, the results of this study are anticipated to provide tangible benefits towards improving the stress tolerance of reproductive organs, and suggest there are merits towards continuing to isolate and functionally characterize ABA-response loci which function specifically during the reproductive development of plants.

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8.0 APPENDICES

APPENDIX 8.1 Supplementary materials and methods associated with PCR, RT-qPCR, microarray and ABA immunolocalization experiments presented in Chapter 3.0.

RNA isolation, reagants and cycling conditions for PCR, RT-qPCR experiments and microarray validation

In the current study RNA was extracted from cauline leaves, inflorescence meristems, developing siliques and seedlings using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) with genomic DNA removed through on column digestion using an RNase free DNase Kit (Qiagen) according to the manufacturer's protocol. Depending on the tissue 1-5µg of total RNA was utilized to synthesize first strand cDNA using a Maxima^{TM} First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada). RT-qPCR studies were carried out using the Sso Fast^{TM} Evagreen$^{®}$ Supermix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) with a final reaction volume of 20µL (10µL master mix, 1µL cDNA, 9µL primer mix). For all RT-qPCR experiments, samples were assayed in triplicate and expression levels were calculated relative to the internal reference gene phosphatase 2A (*PP2A* = At1g13320) subunit (Czechowski et al. 2005). RT-qPCR data was analyzed according to the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Schmittgen and Livak 2008). For RT-qPCR studies a three-step cycling program [5 min at 95°, 40 times (15 sec at 95°, 15 sec at 59-63°, 15 sec at 72°)] was followed by melt curve analysis. During data analysis wells producing abnormal amplification plots or dual melting curves would be eliminated from subsequent analysis. For RT-qPCR

studies and validation of microarrays primers were designed using Primer Quest (Integrated DNA Technologies, IL, USA) and were targeted to regions of genes corresponding to oligonucleotide probe sequences. For PCR studies (end-point) wild-type and *ger5-2* mutants (n = 6 plants per cDNA) were evaluated in parallel on a gradient thermocyler [5 min at 95°, 38 times (45 sec at 95°, 45 sec at 57-61°, 45 sec at 72°), 4 min at 72°] using *Actin* (At3g18780) as an internal reference.

Amplification and labeling of microarrays

During amplification and labeling, aRNA was assessed for integrity and degree of labeling using gel electrophoresis or a Nano-drop spectrophotometer. Arabidopsis oligonucleotide arrays (AROS Version 3.0) were utilized for microarray hybridizations (http://ag.arizona.edu/microarray/methods.html). After re-hydration, UV-crosslinking to immobilize DNA probes, and washing (1% SDS, 100% EtOH) slides were exposed to a pre-hybridization treatment (5x SSC, 0.1% SDS, 0.1 mg/mL^{-1}) at 42°C for 45 minutes. Slides were then washed three times in 0.1x SSC at room temperature (5 minutes), followed by a single wash in water for 30 seconds. Slides were dried in a centrifuge and stored prior to hybridizations.

For hybridization 200-350 ng of Cy5-labelled aRNA or 300-450 ng of Cy3-labelled aRNA was fragmented, denatured, cooled on ice and subsequently combined with hybridization buffer (2.5% formamide, 5x SSC, 0.1 mg/mL^{-1} salmon sperm DNA, 25% KREA*block*) pre-warmed to 42°C. Hybridization solution was applied to oligonucleotide arrays and incubated for 16 hours at 42°C in microarray hybridization chambers. Microarray slides were washed post-hybridization in one wash 2x SSC, 0.1x

SDS at 42°C for 10 minutes, three washes in 1x SSC at room temperature for 5 minutes, and three washes in 0.1x SSC at room temperature for 5 minutes. Microarray slides were then dried by centrifugation and scanned at multiple PMT values (Cy3-532 nm, Cy5-635 nm) on an Axon GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) and analyzed with GenePix® Pro 6.0 (Molecular Devices).

Statistical analysis and validation of microarray

In the current study a mixed model approach developed by Wolfinger et al. (2001) was utilized to assess differences in gene expression amongst seed genotypes or phenotypes as displayed in Fig. 3.8. The experimental design corresponded to three direct comparisons of seed genotypes or phenotypes with 12 Cy3-labelled aRNAs and 12 Cy5- labelled aRNAs hybridized in a dye-swap arrangement against 12 microarray slides (Yang and Speed 2002).

Prior to analysis of the data set missing or erroneous observations were eliminated from subsequent analysis. Studentized residuals were utilized to remove outliers and improve normality of the data set. After $\log_2$ transformation of intensity values, derived from the median spot intensity, expression data were analyzed as a direct comparison between seed genotypes or phenotypes. Data were analyzed within each gene considering genotype and dye as fixed effects and array as a random effect. To reduce false discovery, direct comparisons were only conducted for genes with greater than 6 degrees of freedom. Experiment-wise (all genes combined) type I error rate for P-values was adjusted using the FDR (false discovery rate) option associated with the MULTITEST procedure of SAS, which modifies P-values based on the Benjamini and

Hochberg step-up procedure (Benjamani and Hochberg 1995). FDR adjusted P-values were subsequently used to assess whether differences in gene expression between genotypes or phenotypes were statistically significant. In scenario 1 (FDR ≤ 0.05, 1.25-fold change in gene expression) an FDR of 5% was selected as a cutoff for significance to reduce the number of type 1 errors (false discoveries). However, as discussed in Nettleton (2006), type 2 errors (e.g. truly differentially expressed genes which are not declared to be significantly differentially expressed) can be large when FDR thresholds are kept low. To identify differentially expressed genes contributing towards the phenotype of *ger5-2* mutants (HG versus WT) during seed development, we employed an alternative analysis scenario (FDR ≤ 0.20, 1.1-fold change in gene expression) and accepted a higher rate of type 1 errors (false discoveries) to reduce the number of type 2 errors. Ultimately, genes identified in both analysis scenarios were subsequently examined for microarray validation and follow-up analyses.

ABA immunolocalization

Tissues were fixed in FAA (formaldehyde:acetic acid:ethanol:H_2O) at ratios (v/v) of [10:5:50:35] overnight at 4°C. Samples were subsequently dehydrated in an ethanol series (50, 75, 95, 100, 100%) followed by an ethanol:xylene (v/v) series (3:1, 1:1, 1:3) and 100% xylene (plus solid paraffin chips) overnight. Tissues were then transitioned through xylene:paraffin solutions into pure wax, oriented and allowed to harden before storage at 4°C. Parraffin sections (8 μm) placed on L-polylysine-coated slides were dewaxed with xylene (2 x 10 min), re-hydrated in an ethanol series (100, 100, 95, 85, 50, 25%), followed by two washes in H_2O (5 min each). Slides were then incubated at room

temperature for 15 minutes in FAA buffered with 10% PBS, followed by three washes in PBS (10 min each). Post-fixed slides were incubated in blocking solution (10 mM PBS, pH 7.0, 0.1% Tween-20, 1.5% glycine and 5% (w/v) bovine serum albumin (BSA) for 45 min at RT, then rinsed with low salt buffer I (10 mM PBS, 0.8% NaCl, 0.1% Tween-20, 0.8% BSA). Primary monoclonal antibody against ABA (Phytodetek ABA; Agdia, Elkhart, IN, USA) was diluted (1:100) in PBS and 100 μL added to each slide and incubated in a dark, humid chamber for 24h at 4°C. Slides were then washed twice in high salt buffer (10 mM PBS, 0.1% Tween-20 and 0.8% BSA), followed by wash buffer I and PBS for 10 min each. 100μL of pre-treated secondary antibody (1 mg ml^{-1} anti-mouse immunoglobulin G (IgG) Alkaline Phosphatase (AP) conjugate; Promega, Madison WI, USA) was then added to each slide, with slides subsequently incubated in the darkness for 6 h at room temperature.