*Annual Plant Reviews* (2016) **49**, 253–284
doi: 10.1002/9781119312994.apr0538

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Chapter 9

# GIBBERELLINS AND SEED GERMINATION

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**Abstract:** Gibberellins (GA) promote seed germination, but they are not simple 'Go Ahead' (GA) molecules as the insight gained into the molecular mechanisms underlying their role in seeds appears to be complex. This chapter covers their central role in mediating the environmental and developmental control of seed germination, and how this differs from vegetative growth processes. Spatiotemporal patterns of GA metabolism and GID1-type receptor signalling in the key seed compartments determine tissue interactions and germination timing in response to ambient environmental cues. Gibberellins are key players in seed temperature responses; during thermoinhibition they interact with other hormonal pathways. Allelochemicals such as myrigalone A inhibit seed germination by specific interference with GA metabolism and signalling. This reveals important ecophysiological roles for GAs in seeds and suggests that they are fundamental for studying species adaptation and interaction in natural and agricultural ecosystems upon climate change.

**Keywords:** Abiotic stress and thermoinhibition, allelochemical myrigalone A, *Arabidopsis thaliana*, coat dormancy release, Delay of Germination1 dormancy gene, embryo growth potential, endosperm weakening, GID1-type gibberellin receptor signalling, *Lepidium sativum*, seed gibberellin metabolism

Originally published in *Annual Plant Reviews*, Volume 49: *The Gibberellins*. First Edition.
Edited by Peter Hedden and Stephen G. Thomas.

## 9.1 Introduction

The gibberellin (GA) requirement of seed germination and the importance of *de novo* biosynthesis of bioactive GAs in imbibed seeds were recognized already during the early phase of GA research (e.g. Hashimoto and Yamaki, 1959; Ikuma and Thimann, 1960; Yomo and Iinuma, 1966). The GA requirement for seed germination was also instrumental in screens at the dawn of *Arabidopsis thaliana* (*Arabidopsis*) mutant research: Koornneef and van der Veen (1980) distinguished between 'germinating GA-dwarfs' and 'non-germinating GA-dwarfs' to isolate GA-deficient and GA-insensitive *Arabidopsis* mutants such as *ga1* and *gai* (Koornneef and van der Veen, 1980; Koornneef *et al.*, 1985; Sun *et al.*, 1992; Peng *et al.*, 1997; Koornneef and Meinke, 2010). Treatment with bioactive GA induces the germination of the GA-deficient mutant seeds, and is also used to break seed dormancy and induce seed germination of *Arabidopsis* and other model species, as well as many horticultural species. In this chapter we focus on GA metabolism and signalling during seed germination, with the focus on the non-dormant seed state; for a recent review on the dormant seed state see Graeber *et al.* (2012).

## 9.2 Spatiotemporal expression of gibberellin metabolism during Brassicaceae seed germination

The mature seeds of most angiosperms consist of the embryo surrounded by the diploid maternal testa (seed coat) and the triploid endosperm as distinct covering layers (Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008; Linkies *et al.*, 2010). Seed germination of the Brassicacea *Arabidopsis*, *Lepidium sativum* and *Sisymbrium officinale* progresses from imbibition and swelling by water uptake to the successively visible rupture of the testa and the endosperm (Figure 9.1a) (Liu *et al.*, 2005; Müller *et al.*, 2006; Iglesias-Fernandez and Matilla, 2010). The endosperm rupture is associated with visible radicle protrusion considered as the completion of the seed germination process, and is followed by seedling growth and establishment. Endosperm rupture and radicle protrusion depend on the balance between the weakening of the micropylar endosperm (CAP) surrounding the radicle and the increase in the embryo growth potential required for the elongation of the lower hypocotyl-radicle axis (RAD) (Nonogaki, 2006; Holdsworth *et al.*, 2008; Sliwinska *et al.*, 2009; Linkies and Leubner-Metzger, 2012). The completion of germination by endosperm weakening required for endosperm rupture is promoted by GA, which interacts with other promoting, e.g. ethylene, or inhibiting, e.g. abscisic acid (ABA), hormones, and the weakening also requires proteolysis in the CAP (Linkies *et al.*, 2009, 2010; Morris *et al.*, 2011). Environmental cues, including light, temperature and allelochemicals, mediate their effects, at least in part, by tissue-specific

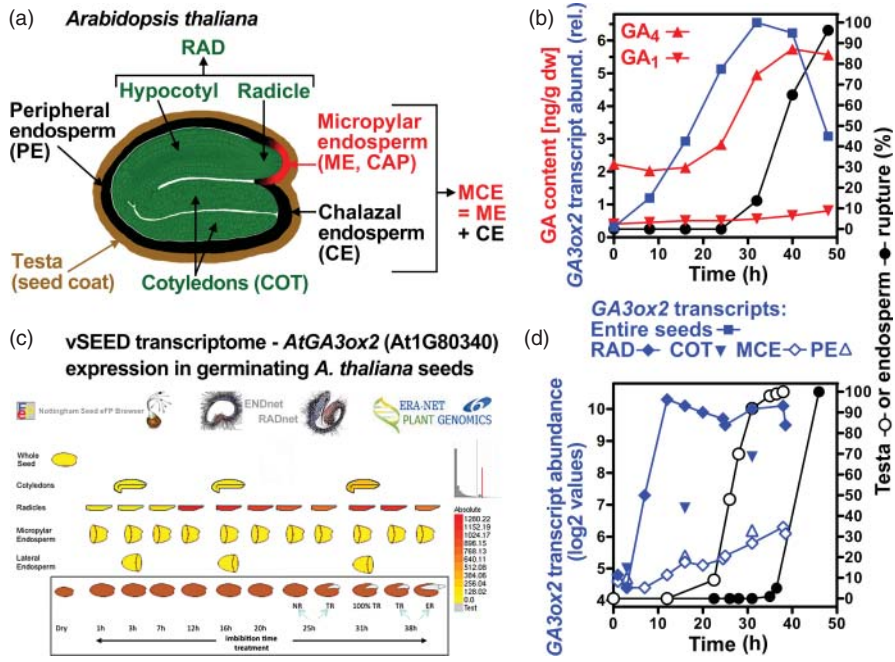



**Figure 9.1** Spatio-temporal expression of the GA-biosynthetic gene *AtGA3ox2* during *Arabidopsis thaliana* seed germination. (a) Structure of a mature seed showing key seed compartments, including RAD (lower one-third of the hypocotyl/radicle axis, embryo growth zone) and CAP/ME (micropylar endosperm). (b) *AtGA3ox2* transcript abundance and bioactive $GA_1$ and $GA_4$ contents during seed germination. Note the increase in $GA_4$ content during late germination. (c) Seed compartment-specific transcriptome analysis during seed germination conducted by the ERA-NET Plant Genomics Consortium vSEED (eFP Browser results from vseed.nottingham.ac.uk). (d) Spatio-temporal *AtGA3ox2* expression during seed germination in relation to the kinetics of testa and endosperm rupture. (Results from (b) Ogawa *et al.*, 2003 and (d) Dekkers *et al.*, 2013.) (See insert for colour representation of this figure.)

alteration of hormone contents and responsiveness (e.g. Kucera *et al.*, 2005; Yamaguchi *et al.*, 2007; Preston *et al.*, 2009; Seo *et al.*, 2009; Weitbrecht *et al.*, 2011; Barua *et al.*, 2012; Oracz *et al.*, 2012). The importance of the tissue-specific nature of this regulation is, for example, evident from the differences in the GA metabolite contents in the *L. sativum* key seed compartments CAP and RAD (Oracz *et al.*, 2012) and from the distinct temporal and spatial pattern of GA biosynthesis and response gene expression in germinating *Arabidopsis* seeds (Ogawa *et al.*, 2003).

The temporal and spatial expression patterns of GA biosynthesis genes have been intensively studied during *Arabidopsis* seed germination (Yamaguchi *et al.*, 2001; Ogawa *et al.*, 2003; Yamauchi *et al.*, 2004; Rieu *et al.*, 2008; Preston *et al.*, 2009) and are summarised in several reviews (Kucera *et al.*,

2005; Yamaguchi *et al.*, 2007; Seo *et al.*, 2009). Bioactive GAs accumulate just prior to radicle protrusion and it seems that GA biosynthesis occurs in two separate locations within the embryo: (1) the early biosynthetic pathway, including the steps catalysed by *ent*-copalyl diphosphate synthase (CPS, the *Arabidopsis GA1* gene At4G02780) and *ent*-kaurene oxidase (KO, the *Arabidopsis GA3* gene At5G25900), in the provascular tissue where *AtCPS* gene promoter activity is localised and (2) the late biosynthetic pathway, including the formation of bioactive GA by GA 3-oxidase, in the cortex and endodermis of the root where *AtGA3ox2* transcripts accumulate and *AtGA3ox2* gene promoter activity was detected. Ogawa *et al.* (2003) also demonstrated that transcript accumulation of *AtGA20ox1* preceded *AtGA3ox2*, and that in addition *AtGA20ox2*, *AtGA20ox3* and *AtGA3ox1* are expressed. However, *GA20ox2* is not highly expressed or induced, while *GA3ox1* displays early expression. Bioactive $GA_4$ was already present in physiologically relevant amounts in the dry, after-ripened seeds used by Ogawa *et al.* (2003) for their transcriptome analysis and further increase in $GA_4$ contents occurs during late germination (Figure 9.1b). Ogawa *et al.* (2003) demonstrated that at least the late GA biosynthesis localises to both compartments, the embryo (radicle plus hypocotyl, RAD) and micropylar endosperm (CAP) during germination, and that within the embryo the early and late biosynthesis pathway may localise to distinct tissues. This implies that intercellular transport of an intermediate of the GA biosynthetic pathway (probably *ent*-kaurene) is required to produce bioactive GA (Kucera *et al.*, 2005).

A recent transcriptome analysis of *Arabidopsis* seed germination (Dekkers *et al.*, 2013) was conducted with a very high temporal and spatial resolution and in relation to the kinetics of testa and endosperm rupture (ER) (Figure 9.1c). The onset of testa rupture (TR) was at around 20 h in the population of imbibed seeds and the completion of TR was at *ca*. 31 h. The onset of endosperm rupture was around 31 h and the completion of ER was at *ca*. 45 h. RNA from defined seed compartments was extracted along the germination time course and, for the 25-h and 38-h time points, non-ruptured and ruptured seeds regarding TR and ER, respectively, were analysed separately. The transcriptome data set of Dekkers *et al.* (2013) reveals two transcriptional phases during germination that are separated by testa rupture. The first phase is marked by large transcriptome changes upon seed imbibition. The second transcriptional phase starts with testa rupture. At the 25-h transition time point (roughly 50% TR, Figure 9.1d) between the two phases these authors analysed the transcriptomes of seeds with and without TR separately. Seed compartment-specific transcriptome analysis was conducted from RAD (radicle plus hypocotyl) and MCE (micropylar endosperm (ME/CAP) plus chalazal endosperm (CE)) for all time points and in addition for COT (cotyledons) and PE (peripheral endosperm) at selected time points (Figure 9.1). The transcripts for the enzymes for *ent*-kaurene formation in the plastid, AtCPS and *ent*-kaurene synthase (AtKS, the *Arabidopsis GA2* gene At1G79460) were more abundant in the embryo compartments

(RAD, COT) compared to the endosperm (MCE, PE). Transcript abundance for genes encoding the enzymes AtKO and *ent*-kaurenoic acid oxidase (the *AtKAO1* and *AtKAO2* genes At1G05160 and At2G32440, respectively; KAO enzymes localise to the endoplasmatic reticulum) which catalyse the formation of $GA_{12}$ (Hedden and Thomas, 2012), were higher in the RAD compared to the other three compartments (Dekkers *et al.*, 2013), supporting the hypothesis that the RAD is the major $GA_{12}$ production site during *Arabidopsis* seed germination. This is in agreement with earlier work demonstrating that the RAD is a major site for the early GA biosynthesis pathway (Ogawa *et al.*, 2003; Kucera *et al.*, 2005; Seo *et al.*, 2009).

The $GA_{12}$ metabolite marks the transition to the late GA biosynthesis pathway, which is localised in the cytosol (Hedden and Thomas, 2012). Of the five *Arabidopsis* GA 20-oxidase genes, three are expressed during seed germination (Yamaguchi *et al.*, 2007). Their transcript expression is induced very early upon seed imbibition and this gene expression differs between the seed compartments (Figure 9.2). *GA20ox1* is mainly expressed in the RAD and there is a striking decrease in the RAD *GA20ox1* transcript abundance associated with the transition between the two transcriptional phases upon testa rupture (see 25 h in Figure 9.2). This decrease in transcript abundance is also evident for *GA20ox2* and *GA20ox3* in the RAD, but is not evident for the MCE. *GA20ox2* transcript expression in the MCE peaked during early germination, and *GA20ox3* transcript expression is highest in the MCE and COT throughout germination (Figure 9.2). The late germination phase, after testa rupture and just prior to endosperm rupture, is therefore characterised by decreased GA 20-oxidase transcript abundances in the RAD, but considerable high *GA20x3* transcript abundance in the MCE. The GA 20-oxidases produce $GA_9$ and $GA_{20}$, the direct precursors of the bioactive $GA_4$ and $GA_1$, respectively (Figure 9.2). The distinct transcript expression patterns of GA 20-oxidases suggest that both the RAD and the MCE are able to produce $GA_9$ and $GA_{20}$ during the early germination phase. Of the four *Arabidopsis* GA 3-oxidases, two are expressed during seed germination (Ogawa *et al.*, 2003; Seo *et al.*, 2009). This earlier finding is in agreement with the results obtained from the vSEED transcriptome published by Dekkers *et al.* (2013), but their spatial resolution provides new insight (Figure 9.2): During the early phase of germination *GA3ox1* and *GA3ox2* transcripts accumulate rapidly in the RAD, and in the late phase of germination in all seed compartments. For *GA3ox1* there is a striking increase in the RAD and MCE transcript abundances associated with the transition between the two transcriptional phases upon testa rupture (see 25 h in Figure 9.2). GA 2-oxidase genes are not expressed during germination, except for the very late phase in which *GA2ox6* is induced in the MCE and *GA2ox2* in the RAD. Taken together, this suggests that the RAD can convert direct precursors into bioactive $GA_4$ and $GA_1$ already during early germination, and that the further increase of bioactive GA is associated with enhanced expression of *GA3ox1* and *GA3ox2* transcript expression in the RAD and MCE upon testa rupture. Bioactive GA production by GA 3-oxidases in

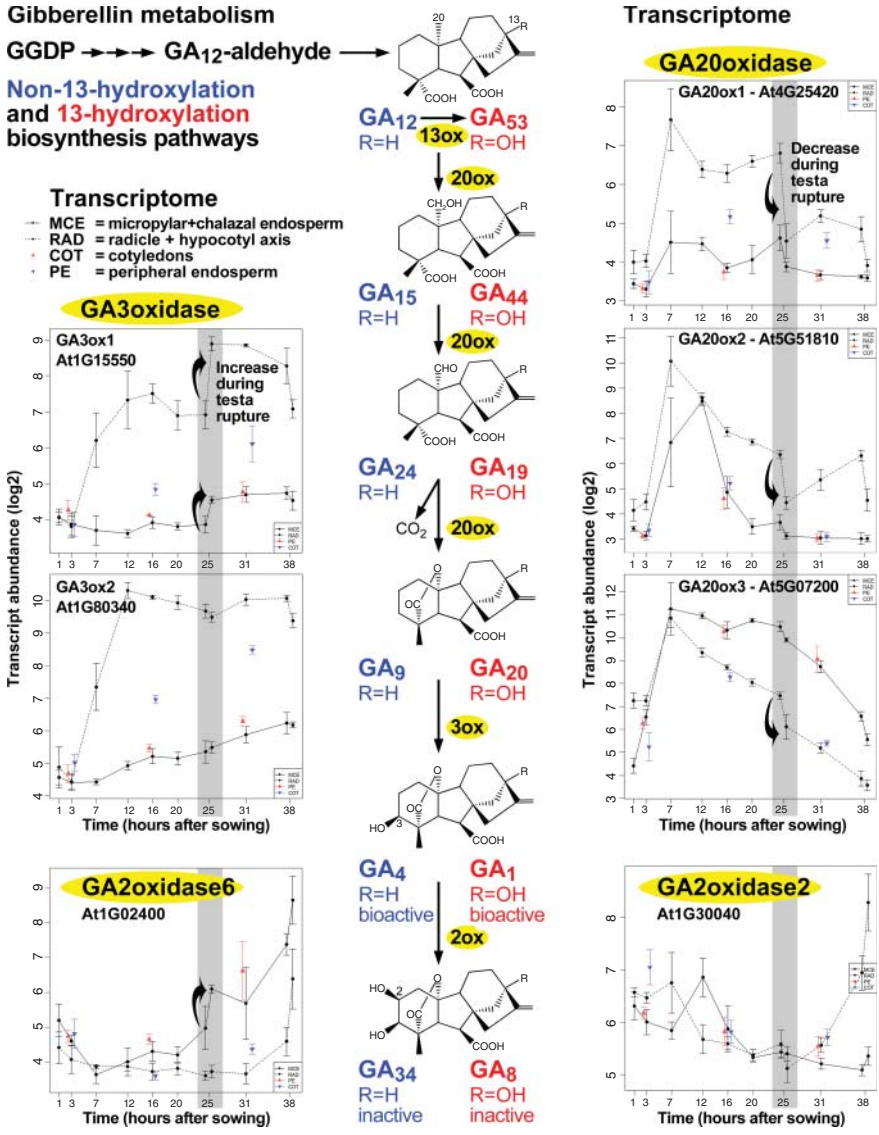
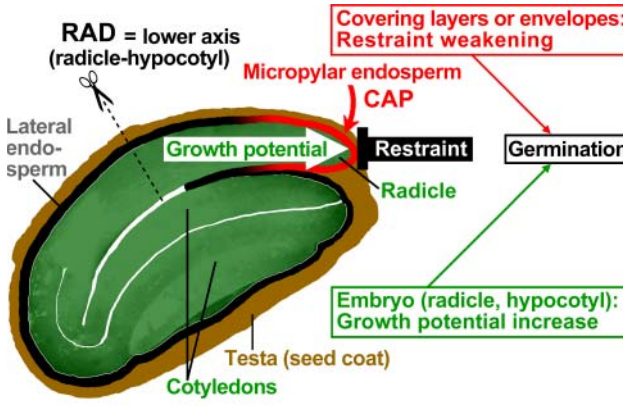



**Figure 9.2** Spatiotemporal gene expression patterns of the *Arabidopsis thaliana* GA metabolic pathway during seed germination. Seed compartment-specific transcriptome results (vseed.nottingham.ac.uk) are presented for the GA 3-, 20-, and 2-oxidase genes, which show major regulation in imbibed seeds. Note that for *GA3ox1* the transcript abundance increases rapidly upon testa rupture (marked as grey area) in RAD and MCE (CAP+CE). The same is evident for *GA2ox6* in MCE, while for the three *GA20ox* genes there is a drastic decrease in transcript abundance in the RAD upon testa rupture. Transcript abundances are from the transcriptome of Dekkers *et al.* (2013) available at vseed.nottingham.ac.uk. The metabolites of the GA pathway are shown with the enzymatic steps indicated.

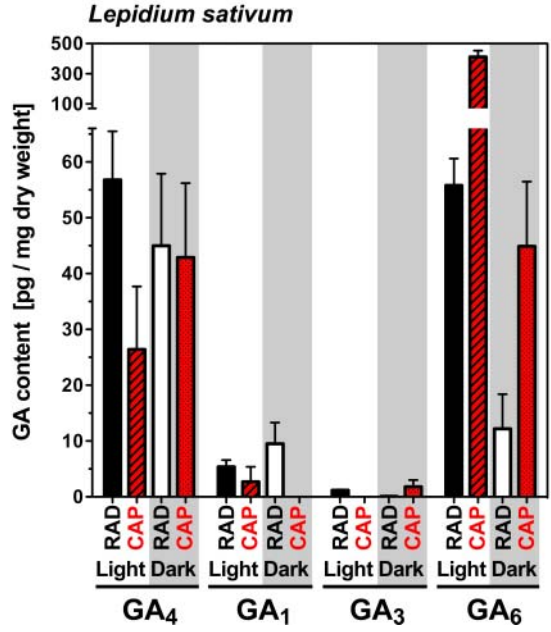
the late germination phase may therefore occur in all seed compartments. Testa rupture marks an important transition during germination and is associated with marked changes in *GA20ox* and *GA3ox* gene expression.

Two roles for bioactive GA during seed germination have been proposed (Kucera *et al.*, 2005; Nonogaki, 2006; Yamaguchi *et al.*, 2007; Linkies and Leubner-Metzger, 2012). Gibberellins increase the growth potential of the embryo to enable RAD elongation by cell growth and are necessary to overcome the mechanical restraint of seed covering layers by weakening of the tissues surrounding the radicle (testa, endosperm CAP). The early induction of GA biosynthesis gene transcripts in the *Arabidopsis* RAD is in concert with the hypothesis that an embryonic GA metabolite and/or bioactive GA itself (the base-level already evident, Figure 9.1b) can diffuse early on during imbibition to the CAP to make it competent for the subsequent weakening during the late germination phase. The transcript expression patterns for GA biosynthesis genes obtained by the vSEED transcriptome analysis (Figures 9.1 and 9.2) suggest that the conversion of inactive precursors into bioactive GAs is further enhanced upon testa rupture by the enhanced expression of *GA3ox* in the embryo (RAD, COT) and the endosperm (MCE, PE), leading to higher $GA_4$ contents (Figure 9.1b) required for endosperm CAP weakening. Indirect evidence for the timing of endosperm weakening of *Arabidopsis* seeds (Debeaujon and Koornneef, 2000; Bethke *et al.*, 2007) is in agreement with this.

Seed germination of GA-deficient mutants depends on the addition of GA to the medium during imbibition (Kucera *et al.*, 2005). *Arabidopsis* GA-deficient *ga1* and ABA-deficient *aba1* mutants, as well as several testa mutants have been studied regarding their GA requirement for dormancy release and seed germination (Debeaujon and Koornneef, 2000; Debeaujon *et al.*, 2000; North *et al.*, 2010). Many testa mutants exhibit reduced seed dormancy due to reduced testa tannin pigmentation. In the presence of GA biosynthesis inhibitors, or when transferred to a GA-deficient background, they are more sensitive to exogenous GA than wild-type. The germination capacity of the *ga1* mutant can be restored, without any contribution of exogenous GA, by removing the surrounding testa and endosperm, or by transferring it to a testa-mutant background. Debeaujon and Koornneef (2000) concluded that dormancy and germination are the consequence of a balance between many promoting and inhibiting factors, such as GA and ABA, which have the embryo and the testa as targets. Their results support the view that the GA requirement for dormancy release and germination is determined by ABA produced in the developing seeds and/or the state of dormancy set by ABA, as well as the amount of ABA produced upon imbibition, especially in dormant seeds. Furthermore, when the testa mutations weaken the restraint to radicle protrusion, the embryo growth potential threshold required for germination is decreased. Therefore, the testa characteristics, embryonic growth potential and embryonic ABA are the determining properties for the GA requirement of *Arabidopsis* seed germination (Kucera *et al.*, 2005).




(a)




(b)

**Figure 9.3** Opposing forces during seed germination and seed compartment-specific analysis of bioactive GAs in *Lepidium sativum*. (a) Seed germination is promoted by the growth potential of the embryonic axis (RAD: radicle plus lower hypocotyl) and inhibited by the restraint of the seed covering layers (testa, endosperm). The completion of germination by radicle emergence and endosperm rupture occurs when the increasing embryo growth potential overcomes the restraint of the micropylar endosperm (CAP). *L. sativum* seeds are a Brassicaceae model to study endosperm weakening as an important developmental process that precedes endosperm rupture. (From Graeber *et al.*, 2010.) (b) The contents of bioactive GAs $GA_4$, $GA_1$, $GA_3$ and $GA_6$, were quantified in both RAD and CAP during late germination (at 15 h, i.e. just prior to endosperm rupture) in light- and dark-imbibed seeds, respectively. (Results compiled from Oracz *et al.*, 2012 and Voegele *et al.*, 2012.)

Gibberellins are important during both the early and the late phases of germination and counteract ABA inhibition. Due to rapid ABA degradation, the ratio of GA/ABA increases *ca.* threefold during early germination and *ca.* 10-fold during late germination of non-dormant *Arabidopsis* seeds (Weitbrecht *et al.* 2011). While for the early germination phase Ogawa *et al.* (2003) did not find altered ABA contents upon treatment of GA-deficient *ga1-3 Arabidopsis* seeds with exogenous GA, Yano *et al.* (2009) found that $GA_4$ contents and *GA3ox1* transcript levels were decreased in ABA-over-producing *cyp707a2 Arabidopsis* seeds (Yano *et al.*, 2009). ABA therefore can inhibit GA biosynthesis during early seed germination. Transcript expression of specific *AtGA20ox*, *AtGA3ox* and *AtGA2ox* genes were demonstrated to be regulated by light (via phytochrome signalling) and temperature (cold-stratification and thermoinhibition) in imbibed *Arabidopsis* seeds (Yamaguchi *et al.*, 2007; Toh *et al.*, 2008; Seo *et al.*, 2006, 2009, 2011; Toh *et al.*, 2012a). *GA20ox* and *GA3ox* genes are induced by red light and cold-stratification. Moist cold-stratification of *Arabidopsis*, i.e. incubation of imbibed seeds at 4 °C in darkness for usually 1-4 days, is routinely used to break dormancy and promote subsequent germination in the light. Yamauchi *et al.* (2004) found that cold stratification is related to the accumulation of *GA20ox* and *GA3ox* transcripts and by increased contents of bioactive GAs (Figure 7c in Weitbrecht *et al.*, 2011). Furthermore, cold-stratification induced a spatial change in *GA3ox1* transcript expression in that it strongly accumulated in the CAP as well as in the RAD (Yamauchi *et al.*, 2004; Weitbrecht *et al.*, 2011). The interaction between the different key seed compartments is therefore of utmost importance for the control of seed germination by GA.

*L. sativum* seeds (Figure 9.3) are similar in structure and physiology to *Arabidopsis* seeds, for example with respect to the presence of a thin living endosperm layer surrounding the mature seed and a two-step germination process with visible testa and endosperm rupture (e.g. Müller *et al.*, 2006; Linkies *et al.*, 2009, 2010; Morris *et al.*, 2011; Linkies and Leubner-Metzger, 2012; Voegele *et al.*, 2012). *L. sativum* seeds, however, differ from *Arabidopsis* seeds in that they are larger, non-dormant, and do not require light for their germination. The larger seed size of *L. sativum* enables the direct biomechanical quantification of the endosperm CAP weakening (Müller *et al.*, 2006; Linkies *et al.*, 2010), as well as seed compartment-specific analysis of transcript and hormone contents, as demonstrated for the CAP and RAD (Linkies *et al.*, 2010; Voegele *et al.*, 2011; Oracz *et al.*, 2012; Voegele *et al.*, 2012). In *L. sativum* seeds the bioactive forms $GA_4$, $GA_6$, $GA_1$ and $GA_3$ were detected in both RAD and CAP during late germination (at 15 h, Figure 9.3b); it is therefore evident that both the 13-hydroxylation and non-13-hydroxylation pathways actively operate in *L. sativum* germinating seeds (Figure 9.4). $GA_4$ as the non-13-hydroxylated product was, on a dry-weight-basis, about twofold more abundant in the RAD compared to the CAP in the light; in dark-imbibed seeds the $GA_4$-contents in these tissues were equal (Figure 9.3b). Compared to $GA_4$, the $GA_1$ (corresponding

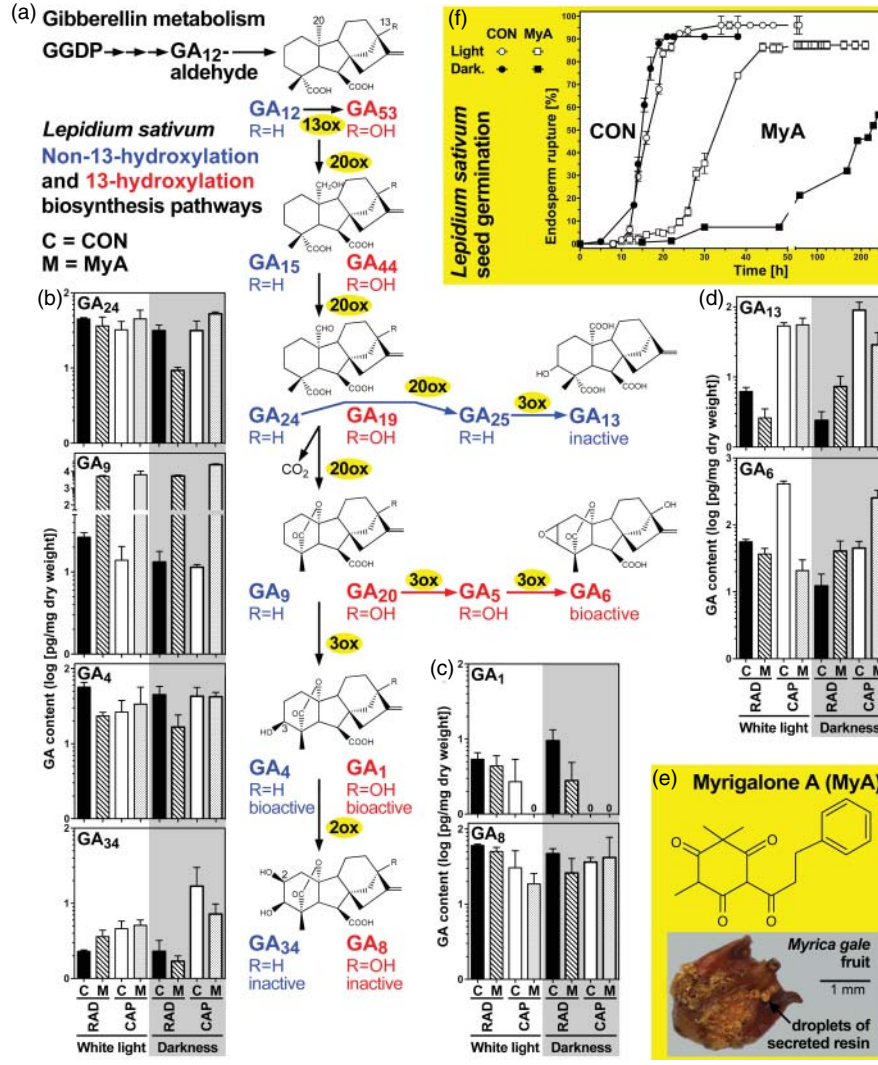



**Figure 9.4** The compartment-specific (RAD, CAP) and light-dependent effect of myrigalone A (MyA) on GA metabolism during germination of *L. sativum* seeds. (a) The non-13-hydroxylation and 13-hydroxylation GA biosynthesis and inactivation pathways and important metabolites detected in *L. sativum* seeds. (b, c, d) Contents of bioactive GAs $GA_1$, $GA_4$ and $GA_6$, and precursors and inactive forms $GA_{24}$, $GA_9$, $GA_{34}$, $GA_8$, $GA_{13}$ quantified in RAD and CAP excised from control (CON) and MyA-treated seeds incubated in continuous light or darkness for 15 h. (e) Chemical structure of MyA and *Myrica gale* fruit with resin droplets containing essential oils. (f) The inhibiting effect of $5 \times 10^{-4}$ M MyA on the germination of *L. sativum* seeds imbibed in continuous light or in darkness. (Compiled results from Oracz *et al.*, 2012 and Voegele *et al.*, 2012.)

13-hydroxylated product) contents were low, and only traces were detected for $GA_3$. The 13-hydroxylated product $GA_6$ was detected at comparable levels to $GA_4$ in RAD and CAP, except for the CAP from light-imbibed seeds, where it accumulated 15-fold compared to $GA_4$.

Based on their work with *L. sativum* seeds, Oracz *et al.* (2012) proposed that in addition to $GA_4$, also $GA_6$ also plays an important role in seed germination. The unambiguous confirmation that the identified compound in *L. sativum* seeds is indeed $GA_6$ requires further analytical work in which verification with another method has to be obtained. Bioactivity of $GA_6$ has been proposed for the induction of flowering and stem extension growth in the grass *Lolium temutentum* (King *et al.*, 2003). GA 3-oxidases, including AtGA3ox1 expressed in *Arabidopsis* seeds (Figure 9.2), and the GA3ox from *Phaseolus* seeds and wheat grains, have been shown to have 2,3-epoxidase activity to produce $GA_6$ from $GA_5$ (Kwak *et al.*, 1988; Zhou *et al.*, 2004; Appleford *et al.*, 2006). The 2,3-epoxide group of $GA_6$ confers its resistance to inactivation by GA 2-oxidases. $GA_6$ may also serve for transport or accumulation as it is a stable bioactive GA (Pimenta Lange and Lange, 2006; Yamaguchi, 2008). As $GA_6$ accumulates specifically in the CAP of *L. sativum* seeds, it was proposed that it contributes, together with $GA_4$, to the endosperm CAP weakening (Oracz *et al.*, 2012; Voegele *et al.*, 2012; Graeber *et al.*, 2014). From these compartment-specific measurements (Figure 9.3b) the combined bioactive $GA_4$ and $GA_6$ concentrations can be estimated as *ca.* 20 nM in the RAD and *ca.* 125 nM in the CAP of *L. sativum* seeds imbibed in continuous light.

Figure 9.4 shows that $GA_8$ and $GA_{13}$ were abundant inactive metabolites in *L. sativum* seeds (Oracz *et al.* 2012; Voegele *et al.* 2012). $GA_8$ is the 2β-hydroxylated inactivation product from $GA_1$ and its accumulation explains the low $GA_1$ contents. $GA_{13}$ contents were 9- and 37-fold in CAP compared to RAD of seeds imbibed in continuous light and in darkness, respectively (Figure 9.4). It is known as an unusual product found in developing seeds requiring atypical GA20ox and GA3ox enzymes, its function is unknown and it is inactive (Pimenta Lange and Lange, 2006). Work on developing pumpkin seeds demonstrated that a GA 3-oxidase which converts $GA_9$ to $GA_4$, also converts $GA_{25}$ to $GA_{13}$ (Frisse *et al.* 2003). In support for this, $GA_{13}$ contents were reduced in seedlings of the *A. thaliana ga3ox1* mutant (Talon *et al.*, 1990). As $GA_{13}$ can bind to GA 2-oxidases, its accumulation in *L. sativum* CAP tissues may serve as GA2ox activity inhibitor and thereby prevent $GA_4$ inactivation. Recent work by Nomura *et al.* (2013) identified AtCYP714A1 (At5G24910) as an enzyme which converts $GA_{12}$ to biologically inactive 16-carboxylated $GA_{12}$. During the early germination of *Arabidopsis* seeds, transcripts of AtCYP714A1 were highly abundant in the MCE and PE, but declined rapidly during the late phase and were low in the RAD and COT (vseed.nottingham.ac.uk, Dekkers *et al.* 2013). Epoxidation and hydroxylation, including those catalysed by GA 3-, GA 2- and GA 13-oxidases, mediate the fine-tuning of late GA metabolism (Hedden and Thomas, 2012; Magome *et al.*, 2013; Nomura *et al.*, 2013) and for germinating

seeds this is also evident from the compartment-specific enzyme transcript and GA metabolite contents (Figures 9.1 to 9.4).

A recent publication by Graeber *et al.* (2014) shows that the dormancy gene *DOG1 (DELAY OF GERMINATION1)* controls dormancy by setting the optimal ambient temperature window for germination. This is achieved by temperature-dependent alteration of GA metabolism, while ABA metabolism is not appreciably affected. Over-expression of *AtDOG1* in transgenic *L. sativum* seeds leads to a generally enhanced GA biosynthesis by up-regulated *KAO* expression and an altered temperature-responsiveness of *GA20ox* expression. These findings suggest that DOG1 interferes with the negative feedback regulation of *GA20ox* in a temperature-dependent manner (Graeber *et al.*, 2014). The altered GA metabolism leads in turn to altered expression of genes required for the biomechanical weakening of the coats encasing the embryo. The over-expression of *DOG1* does not affect the embryo growth potential. Regulation of GA metabolism is therefore a key process in the DOG1-mediated conserved coat-dormancy mechanism of seeds.

## 9.3 Gibberellin signalling and seed germination

### 9.3.1 The GID1ac and GID1b pathways in seeds

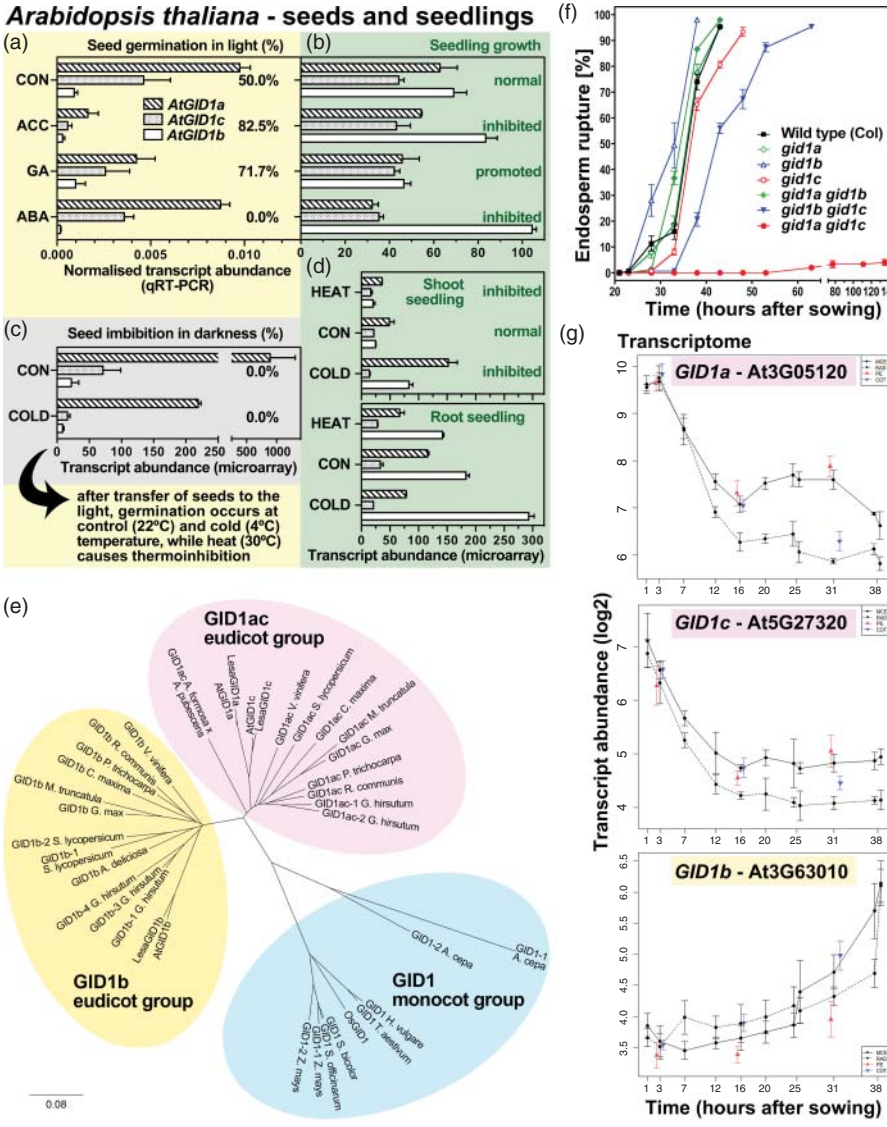
The discovery that *GID1 (GIBBERELLIN INSENSITIVE DWARF 1)* encodes a soluble GA receptor in rice (Ueguchi-Tanaka *et al.*, 2005) and subsequent work on the GID1 receptors of other species (e.g. Griffiths *et al.*, 2006; Iuchi *et al.*, 2007; Willige *et al.*, 2007; Voegele *et al.*, 2011; Hauvermale *et al.*, 2012) has considerably advanced our understanding of the GA signal transduction cascade. The DELLA proteins are repressors of GA signalling and repress the GA responses, e.g. seedling growth and seed germination, by their interaction with transcription factors (see Chapter 7). Bioactive GAs promote DELLA repressor degradation in an environmentally and developmentally dependent manner. The GA-dependent alleviation of the DELLA-imposed repression is achieved by GID1 binding GA, which in turn enables GID1-DELLA complex formation resulting in DELLA recognition, ubiquitination, followed by 26S-proteasome-mediated DELLA degradation (see Chapter 6). Despite evidence, summarised in Chapter 1, indicating that GA-mediated induction of α-amylase genes in the aleurone of germinating cereal grains involves a plasma membrane receptor, recent work by Yano et al. (2015) strongly suggests that GA signalling in the rice aleurone utilises only OsGID1. GA signalling in the cereal aleurone has been reviewed by Sun and Gubler (2004) and is also discussed in Chapter 7.

*Arabidopsis* contains three GID1 receptors: AtGID1a, AtGID1b and AtGID1c. Figure 9.5 shows that environmental, hormonal and developmental cues affect expression of the three *AtGID1* genes. Gibberellins promote

seedling growth and seed germination, while ethylene inhibits seedling growth, but promotes seed germination (Linkies *et al.*, 2009; Voegele *et al.*, 2011; Linkies and Leubner-Metzger, 2012). In germinating *Arabidopsis* seeds, treatment with GA or with ACC (1-aminocyclopropane-1-carboxylic acid, the direct biosynthetic precursor of ethylene) reduced *AtGID1* transcript expression (Figure 9.5a). In contrast to seeds, this GA- and ethylene-mediated negative regulation was not evident in growing seedlings (Figure 9.5b). This effect might have been caused by seedling saturation by GA (in contrast to seed). Furthermore, the response to treatment with ABA differed for AtGID1b between seeds and seedlings, in that ABA down-regulates *AtGID1b* transcript expression in seeds while it up-regulates it in seedlings. Different regulation of *AtGID1* transcripts in seeds and seedlings in response to ambient temperature is also evident (Figure 9.5c,d). For cold temperature this relationship is, however, complicated by the fact that *Arabidopsis* seeds required cold-stratification and light for dormancy release and/or germination (Figure 9.5c). An important point from these examples is that GA signalling in seeds is distinct from seedlings and that AtGID1 expression is part of this distinct regulation.

Voegele *et al.* (2011) showed by molecular phylogenetic analysis of the angiosperm GID1 receptor family individual clustering of GID1 proteins into three distinct groups: eudicot GID1ac, eudicot GID1b and monocot GID1 (Figure 9.5e). It has been demonstrated that the individual GID1 receptors of *Arabidopsis* (AtGID1a, AtGID1b, AtGID1c) display partial redundancy and have functional specificities for regulating the GA-responsiveness of different developmental processes (e.g. Griffiths *et al.* 2006; Iuchi *et al.* 2007; Voegele *et al.*, 2011). The distinction between two eudicot groups (GID1ac and GID1b) and the monocot group is also supported by biochemical evidence in studies with the three *Arabidopsis* GID1 receptors: Nakajima *et al.* (2006) showed that AtGID1a and AtGID1c bind $GA_4$ and $GA_3$ with lower affinity compared to AtGID1b. It should be mentioned, however, that less work has been carried out on monocot species seed germination so far, and that the degree of partial redundancy between the two eudicot pathways may differ, depending on the developmental process. The regulation of the GID1 pathways and its roles during seed germination differ from other developmental processes and may be restricted to the Brassicaceae family.

Voegele *et al.* (2011) analysed the *Arabidopsis* knockout mutants for the three AtGID1 receptors and showed that the AtGID1b receptor is not able to compensate for the seed germination phenotype of the *gid1agid1c* double mutant (Figure 9.5f). Thus GA signalling via the GID1ac receptors is required for seed germination. In contrast, the AtGID1a and AtGID1c receptors are partially redundant and can substitute for AtGID1b. Based on the seed germination phenotypes of the *Arabidopsis* knock-out mutants and the ABA-related *LesaGID1ac* and *LesaGID1b* transcript expression patterns in the micropylar endosperm (CAP) and the RAD of *L. sativum*, the GID1c receptors may have a major influence on seed germination. Transcript


Arabidopsis thaliana - seeds and seedlings
(a)
Seed germination in light (%)
CON
ACC
GA
ABA
50.0%
82.5%
71.7%
0.0%
AtGID1a
AtGID1c
AtGID1b
Normalised transcript abundance (qRT-PCR)
(b)
Seedling growth
normal
inhibited
promoted
inhibited
(c)
Seed imbibition in darkness (%)
CON
COLD
0.0%
0.0%
Transcript abundance (microarray)
after transfer of seeds to the light, germination occurs at control (22ºC) and cold (4ºC) temperature, while heat (30ºC) causes thermoinhibition
(d)
HEAT
CON
COLD
Shoot seedling
inhibited
normal
inhibited
Root seedling
HEAT
CON
COLD
Transcript abundance (microarray)
(e)
GID1ac eudicot group
GID1b eudicot group
GID1 monocot group
0.08
(f)
Endosperm rupture [%]
Wild type (Col)
gid1a
gid1b
gid1c
gid1a gid1b
gid1b gid1c
gid1a gid1c
Time (hours after sowing)
(g)
Transcriptome
GID1a - At3G05120
GID1c - At5G27320
GID1b - At3G63010
Transcript abundance (log2)
Time (hours after sowing)

abundances in the order *AtGID1a* > *AtGID1c* > *AtGID1b* were evident in dry and imbibed seeds (Figure 9.5g). Their spatiotemporal transcript expression patterns during germination were similar for *AtGID1a* and *AtGID1c*, but distinct for *AtGID1b*: While the transcript abundance of *AtGID1b* increased during germination, those for *AtGID1a* and *AtGID1c* decreased (Figure 9.5g). The hypothesis of the different degrees of importance of the individual Brassicaceae *GID1* genes during seed germination, with *GID1b* being distinct from *GID1ac*, is also supported by the stronger GUS staining in seeds of *AtGID1ac*-promoter reporter gene lines compared to *AtGID1b* (Voegele *et al.*, 2011). This finding is further supported by the fact that Griffiths *et al.* (2006) found negative feedback-regulation by GA for all three *GID1* transcripts in *Arabidopsis* seedlings, while Voegele *et al.* (2011) demonstrated from transcript analyses and GUS reporter line staining results, combined with *in silico* analysis using the eFP browser, that down-regulation by GA during the germination of unstratified *Arabidopsis* seeds was evident only for the *AtGID1a* and *AtGID1c* transcripts, but not for the *AtGID1b* transcripts. An alternative interpretation might be a higher GA sensitivity

←

**Figure 9.5** The effect of environmental, hormonal and developmental conditions on the expression of the three GID1 GA receptor genes in *Arabidopsis*. (a) *AtGID1a*, *AtGID1*b and *AtGID1c* transcript abundances were determined by qRT-PCR in whole seeds during germination. Seeds were incubated for 30 h in continuous white light without (CON) or with 1mM ACC, 10 μM $GA_{4+7}$ (GA) or 1 μM ABA added; ACC results (Voegele, unpublished results), and GA/ABA results (Voegele *et al.*, 2011) presented for comparison, are from the same experiment. Expression values relative to validated constitutive transcripts are presented as mean values ±SE of 4 × >1000 seeds. The percentage of endosperm rupture of the individual seed populations is indicated next to the bars. (b) GID1 transcript abundances from microarray data of *Arabidopsis* seedlings (Kilian *et al.* 2007). The effect of the treatment on seedling growth is indicated next to the bars. Microarray data for *Arabidopsis* were obtained from the BAR eFP-Browser (Winter *et al.*, 2007; Bassel *et al.*, 2008). (c) *GID1* transcript abundance from microarray data of *Arabidopsis* seeds imbibed in darkness at 22 °C (CON) and 4 °C (COLD) (Yamauchi *et al.*, 2004). Note that dormancy release by cold-stratification causes the indicated germination responses at different temperatures in the light. (d) *GID1* transcript abundance from microarray data from *Arabidopsis* seedling shoots and roots, grown at 30 °C (HEAT), 22 °C (CON), 4 °C (COLD) (Kilian *et al.* 2007). (e) The molecular phylogenetic analysis of the angiosperm GID1 receptor family individual clustering of GID1 proteins into three distinct groups: eudicot GID1ac, eudicot GID1b and monocot GID1; AtGID1 = *Arabidopsis thaliana* GID1, LesaGID1 = *Lepidium sativum* GID1, OsGID1 = *Oryza sativa* GID1 (from Voegele *et al.*, 2011). (f) Time-course analysis of endosperm rupture of after-ripened seeds of *Arabidopsis* single (*gid1a*, *gid1b*, *gid1c*) and double (*gid1agid1b*, *gid1bgid1c*, *gid1agid1c*) mutants imbibed at 24 °C in continuous light without preceding stratification (from Voegele *et al.*, 2011). (g) The spatiotemporal gene expression patterns of A*tGID1a*, *AtGID1b* and *AtGID1c* during seed germination. Note that *GIDac* exhibit a similar pattern, which is distinct from *GID1b*. (Transcript abundances are from the transcriptome of Dekkers *et al.*, 2013 available at vseed.nottingham.ac.uk.)

of the negative feedback mechanism on *AtGID1b* transcript abundance compared to *AtGID1a* and *AtGID1c* as proposed by Iuchi *et al.* (2007). The endogenous GA content (Figure 9.1b) would then already be sufficient to decrease *AtGID1b* transcript abundance. In support of this proposal, the GA-triggered negative feedback loop on *AtGID1a* and *AtGID1c*, but not on *AtGID1b*, was also evident in imbibed *ga1-3 Ler* seeds (Ogawa *et al.*, 2003). Furthermore, during *L. sativum* seed germination, a GA-triggered negative feedback loop in the CAP and the RAD was only evident for the *LesaGID1a* and *LesaGID1c* transcripts (Voegele *et al.*, 2011). This strongly suggests that a GA-triggered negative feedback loop during seed germination exists for *GID1a* and *GID1c* in Brassicaceae seeds, while *GID1b*-type transcripts are not down-regulated. In addition, in both species expression patterns are similar regarding transcript abundance during early germination (8 h in *L. sativum* vs. 30 h *Arabidopsis*), in which the *GID1ac* transcript levels are usually higher compared to *GID1b*, and no significant regulation by ABA during early germination takes place. However, *GID1b* with its unique binding activity to $GA_4$ and its pH dependence is together with *GID1ac* required for Brassicaceae seed germination although their expression patterns differ spatially, temporally and hormonally. Taken together, the GID1ac and GID1b receptor groups are both important for proper seed germination, but both groups play distinct roles during this process.

### 9.3.2 DELLA proteins and seed germination

All three *Arabidopsis* GID1 receptors can interact with all five *Arabidopsis* DELLA repressor (GAI (GA-insensitive), RGA (repressor-of-ga1-3), RGL1 (RGA-like1), RGL2, RGL3) targets (Nakajima *et al.*, 2006; Willige *et al.*, 2007; Suzuki *et al.*, 2009). In an evolutionary context a separation between an GID1ac-type (interacting preferentially with the RGL1/RGL2/RGL3 group of DELLA repressors) and a GID1b-type (interacting preferentially with the GAI/RGA group of DELLA repressors) pathway (Suzuki *et al.*, 2009) hints to a greater specialization of eudicot GID1-mediated GA signalling, while such a partial functional separation has not occurred within the monocots. In monocot species, solely one group of GID1 receptors has been identified (Figure 9.5e). This might be associated with fewer DELLA proteins present in monocot: SLR1 in *Oryza sativa*, SLN1 in *Hordeum vulgare*, D8 and D9 in *Zea mays* (Peng *et al.*, 1999; Ikeda *et al.*, 2001; Gubler *et al.*, 2002; Weston *et al.*, 2008). Differential expression and distinct patterns of degradation of different DELLA repressors has been shown in seeds (Bassel *et al.*, 2004; Piskurewicz *et al.*, 2008, Piskurewicz and Lopez-Molina. 2009; Piskurewicz *et al.*, 2009; Voegele *et al.*, 2011; Ariizumi *et al.*, 2013; Chandler and Harding, 2013). It has been suggested that RGL1 possesses a more major role in seed germination than do GAI and RGA, but that RGL2 is the most crucial regulator of seed germination in *Arabidopsis* in response to GA (reviewed by Kucera *et al.*, 2005). Light- and GA- independent seed germination can be obtained by the

loss of function of four *DELLA* genes (*RGL2*, *RGL1*, *RGA* and *GAI*). From former findings it has been proposed that RGA and GAI destabilization or inactivation in seeds might be triggered by GA. This fact supports the view that DELLA repressors integrate environmental and endogenous cues with the seed germination regulation (reviewed by Kucera *et al.*, 2005).

The role of light (via phytochrome) in the DELLA-mediated seed germination response has been elucidated in *Arabidopsis* (Oh *et al.*, 2006, 2007; Holdsworth *et al.*, 2008; Penfield and Hall, 2009). Red-light activated phytochrome (Pfr form) decreases PIL5 (phytochrome-interacting factor3-like5; bHLH protein) activity, which enhances germination. In darkness, PIL5 directly binds to the *RGA* and *GAI* gene promoters and stimulates DELLA repressor expression, as well as indirectly increasing *GA2ox* gene expression (GA inactivation) and expression of ABA biosynthetic genes. In addition, the GA biosynthetic genes *GA20ox* and *GA3ox* are down-regulated by PIL5. Penfield *et al.* (2005) found that the transcription factor SPATULA (SPT), together with PIF3 and PIL5, is involved in mediating the light and temperature responses of seed germination; and in dormant seeds this involves down-regulation of *GA3ox*. Moreover, PIL5 stimulates the expression of *SOMNUS* (*SOM*), encoding a CCHH-type zinc finger protein (Kim *et al.*, 2008; Park *et al.*, 2011). The role of PIL5 is significant, provided GA synthesis is sufficiently high; otherwise, high GAI and RGA protein levels persist to block germination. SOM represses germination by down-regulating and stimulating GA and ABA synthesis gene expression, respectively. ABA-INSENSITIVE3, ABA-INSENSITIVE5 and DELLAs interact to activate the expression of SOMNUS and other high-temperature-inducible genes in imbibed *Arabidopsis* seeds (Lim *et al.*, 2013). The GA-signalling repressors RGL2 and RGL3 inhibit *Arabidopsis* seed germination by stimulating ABA biosynthesis and ABI5 activity (Piskurewicz *et al.*, 2008; Piskurewicz and Lopez-Molina, 2009) and the inhibition of cell-wall remodelling protein gene expression (Morris *et al.*, 2011; Voegele *et al.*, 2011; Stamm *et al.*, 2012). RGL2 transcript and protein levels dominate relative to that of other DELLA factors such as RGA and GAI, whereas RGL3 transcripts could only be observed in the absence of RGL2 (as in an rgl2 mutant background) (Piskurewicz *et al.*, 2008, Piskurewicz and Lopez-Molina, 2009). Piskurewicz and Lopez-Molina (2009) reported that RGL2 represses testa rupture in response to changes in GA and ABA levels. The testa rupture in rgl2 mutants is insensitive to low GA or high ABA conditions. Far-red light inhibits germination through DELLA-dependent stimulation of ABA synthesis and the transcription factor ABA-INSENSITIVE 3 (ABI3) (Piskurewicz *et al.*, 2009). In white light, transgene expression of *GAI* and *RGA* driven by the *RGL2* promoter can substitute for *RGL2* to promote ABA synthesis and repress germination, consistent with the recent findings with RGL2. The three DELLA factors repress testa rupture, whereas ABI3 blocks endosperm rupture (Piskurewicz *et al.*, 2009). In the shade, endospermic ABA opposes phyA signalling through the transcription factor ABI5, which shares with the bHLH transcription

factor PIF1 several target genes that negatively regulate germination in the embryo. ABI5 enhances the expression of phytochrome signalling genes *PIF1*, *SOMNUS*, *GAI* and *RGA*, but also of ABA and GA metabolic genes (Lee *et al.*, 2012). CHOTTO1, a putative double APETALA2 repeat transcription factor, acts downstream of ABA to repress GA biosynthesis during seed germination (Yano *et al.*, 2009). ABI4 is implicated in the regulation of the ABA-GA balance and reserve mobilisation in seeds (Penfield *et al.*, 2006; Cantoro *et al.*, 2013; Suh *et al.*, 2013). Park *et al.* (2013) isolated from seedling tissue DELLA-interacting RING domain proteins, BOI-RELATED GENE1 (BRG1), BRG2 and BRG3 (collectively referred to as BOIs). Single *Arabidopsis* mutants of each *BOI* gene did not significantly influence GA responses, but the *boi* quadruple mutant (*boiQ*) showed a higher seed germination frequency in the presence of paclobutrazol (GA inhibitor). They propose that the DELLA and BOI proteins inhibit GA responses by interacting with each other, binding to the same promoters of GA responsive genes, and down-regulating these genes.

## 9.4 Gibberellin and abiotic stress factors: thermoinhibition of seed germination

Thermoinhibition is the failure of seeds to germinate at high imbibition temperatures. The delayed or severely inhibited seed germination by thermoinhibition is mediated by complex alterations of hormone metabolism and signalling (Watt *et al.*, 2011; Linkies and Leubner-Metzger, 2012; Toh *et al.*, 2012a; Huo *et al.*, 2013). The research group of Kent Bradford studied the mechanisms of lettuce thermoinhibition by comparing the germination of *Lactuca sativa* cv. Salinas (*'L. sativa* Sal'), a cultivated variety exhibiting thermoinhibition, with *Lactuca serriola* UC96US23 (*'L. serriola* UC'), a wild progenitor accession exhibiting thermotolerance, at different temperatures (Argyris *et al.*, 2005, 2008, 2011; Schwember and Bradford, 2010a, 2010b; Huo *et al.*, 2013). While both lettuce genotypes germinated when imbibed in the light at 20 °C, only *L. serriola* UC was able to germinate at 35 °C (Figure 9.6a). *L. serriola* UC is thermotolerant until *ca.* 38 °C, while thermoinhibition of *L. sativa* Sal starts above *ca.* 25 °C (Argyris *et al.*, 2008; Huo *et al.*, 2013). The switch to thermoinhibition occurs within a narrow temperature window of 2–3 °C, and different lettuce genotypes differ in the temperature for this switch, while the hormonal mechanisms causing thermoinhibition above this temperature appear to be similar. The lettuce ABA biosynthesis gene *LsNCED4* (encoding a 9-*cis*-epoxycarotenoid dioxygenase) was identified as a major quantitative trait gene (QTG) by conducting quantitative trait loci (QTL) analysis of recombinant inbred line (RIL) populations derived from a cross between *L. sativa* Sal x *L. serriola* UC (Argyris *et al.*, 2008, 2011; Huo *et al.*, 2013). In agreement with a major role of the *LsNCED4* QTG

during thermoinhibition, its expression was up-regulated and the seed ABA contents and sensitivities were increased at temperatures causing thermoinhibition. Argyris *et al.* (2008) conclude that the temperature sensitivity of *LsNCED4* expression may determine the upper temperature limit for lettuce seed germination. Figure 9.6c summarises their findings about how high temperature affects the expression of hormone-related genes and demonstrates that the up-regulation of ABA biosynthesis is associated with down-regulation of GA- and ethylene-related biosynthesis genes. Huo *et al.* (2013) demonstrated that silencing of the *LsNCED4* gene in transgenic lettuce seeds altered the expression of genes involved in ABA, GA and ethylene biosynthesis and signalling pathways. The hormonal cross-talk in lettuce seed thermoinhibition (Figure 9.6c) seems therefore to be mediated by indirect (elevated ABA contents due to up-regulated *LsNCED4* gene expression) and direct effects of the high temperature. These findings are in agreement with the knowledge that not an individual hormone, but the content ratios between promoting and inhibiting hormones (such as GA/ABA) combined with the seed sensitivities (interaction between the endogenous contents and the state of the corresponding signalling pathways) determine the seed responses (Kucera *et al.*, 2005; Holdsworth *et al.*, 2008; Linkies and Leubner-Metzger, 2012). Work with *Arabidopsis* supports the view that the hormonal interactions described for lettuce (Figure 9.6c) constitute a conserved mechanism for seed thermoinhibition (Gonai *et al.*, 2004; Toh *et al.*, 2008; Seo *et al.*, 2009; Toh *et al.*, 2012a; Toh *et al.*, 2012b). Regarding signalling components, increased expression of DELLA repressors, the ethylene-signalling component CTR1, as well as the ABA-related components ABI3, ABI4, ABI5, SNF4 (Figure 9.6c), and the transcription factor FUSCA3 have been proposed to be involved (Chiu *et al.* 2012).

In agreement with a major role for GAs in counteracting the ABA inhibition and in alleviating thermoinhibition, high temperature inhibited the expression of *GA3ox* genes in imbibed seeds of lettuce (Argyris *et al.*, 2008) and *Arabidopsis* (Toh *et al.*, 2008). In contrast to thermoinhibited *L. sativa* Sal seeds, *LsGA3ox1* expression was evident in the thermotolerant *L. serriola* UC seeds imbibed at 35 °C in continuous light (Figure 9.6a); similar results were obtained for *LsGA3ox2*. It was demonstrated for *Arabidopsis* that thermoinhibition is associated with decreased expression of *AtGA3ox2* and *AtGA3ox1* and of $GA_4$ and $GA_1$ contents, and, in agreement with a role of the decreased seed GA contents, can be alleviated by treatment with bioactive GA (Figure 9.6b).

*Arabidopsis* seed $GA_4$ and $GA_1$ contents are strongly influenced by imbibition temperature: compared to 22 °C they decreased in response to thermoinhibition (34 °C in the light) and, as described above, increased during cold-stratification (4 °C in the dark) (Yamauchi *et al.*, 2004; Toh *et al.*, 2008) (Figure 9.6b). This finding is consistent with the observed up-regulation of *AtGA3ox1* expression by low temperature. Expression of *AtGA3ox2*, the major *AtGA3ox* gene induced during seed germination, is, however, not

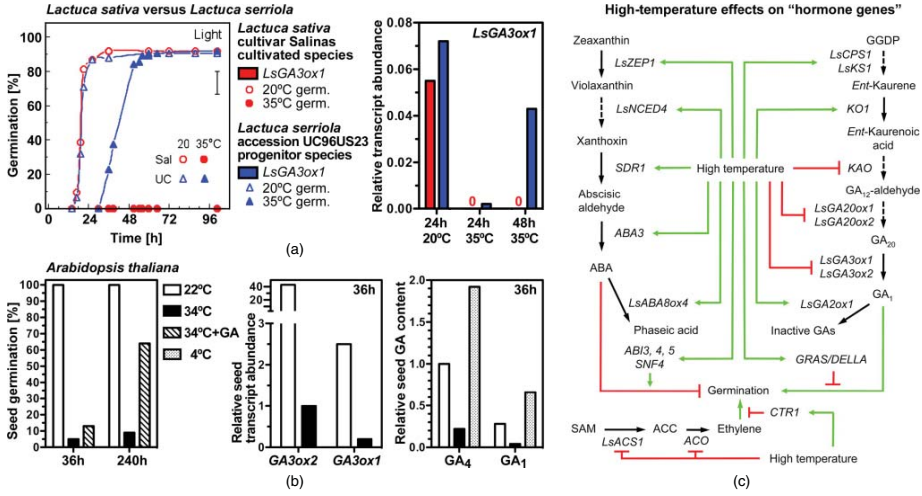



**Figure 9.6** Hormonal aspects of thermoinhibition of lettuce and *Arabidopsis* seed germination. (a) The effect of supra-optimal temperature on germination (*left*) and *LsGA3ox1* gene expression (*right*) during germination of *Lactuca sativa* cv. Salinas (cultivated lettuce) and *Lactuca serriola* UC96US23 (thermotolerant lettuce). Note that compared to 20 °C, thermoinhibion of *L. sativa* occurs at 35 °C and is associated with inhibited *LsGA3ox1* expression, while the gene is expressed in thermotolerant *L. serriola* seeds (results from Argyris *et al.*, 2008). (b) Thermoinhibition of *Arabidopsis* seeds imbibed in the light occurs at 34 °C and is alleviated by GA addition to the medium (*left*). The effect of temperature on the expression of the GA biosynthetic genes *AtGA3ox1* and *AtGA3ox2* and on the seed bioactive $GA_1$ and $GA_4$ contents is shown (*right*). Note that thermoinhibition is associated with a decrease, while cold-stratification (4 °C) is associated with an increase in the seed GA contents (results from Toh *et al.*, 2008 and from Yamauchi *et al.*, 2004). (c) Promoting (arrows) and inhibiting (bars) effects of high temperature on metabolism and signalling genes of hormones (GA, ABA and ethylene) involved in lettuce seed germination (from Argyris *et al.*, 2008). (See insert for colour representation of this figure.)

up-regulated during cold-stratification, while high temperature inhibited the expression of both *AtGA3ox2* and *AtGA3ox1* (Figure 9.6b, Toh *et al.*, 2008). Light (via phytochrome) as a required factor for *Arabidopsis* seed germination is also required for *AtGA3ox2* expression (Yamaguchi *et al.*, 2007). This provides a possible explanation for the finding that *AtGA3ox2* is not up-regulated as the cold-stratification is conducted in the dark. Light also promotes germination of lettuce seeds, in which *LsGA3ox2* expression is not induced in the dark at any temperature, while low-level expression of *LsGA3ox1* is induced at 20 °C in both genotypes, and at 35 °C only in thermotolerant *L. serriola* UC seeds, but not in thermoinhibited *L. sativa* Sal seeds (Argyris *et al.*, 2008).

Thermoinhibition also suppressed induction of *AtGA20ox2* and *AtGA20ox3* during the early imbibition of *Arabidopsis* seeds (Toh *et al.*, 2008), as it did for the *LsGA20ox1* and *LsGA20ox2* genes during the early imbibition of *L. sativa* Sal seeds (Argyris *et al.*, 2008). In contrast to the thermoinhibited *L. sativa* Sal seeds, *LsGA20ox1* and *LsGA20ox2* gene expression was evident at 35 °C in thermotolerant *L. serriola* UC seeds. Interestingly, except for *LsKAO*, early GA biosynthetic genes such as *LsCPS1*, *LsKO1*, and *LsKS1*, were expressed in thermoinhibited *L. sativa* Sal seeds. Also, there was no clear pattern for the regulation of *GA2ox* genes during *Arabidopsis* and lettuce thermoinhibition (Argyris *et al.*, 2008; Toh *et al.*, 2008), except that at 35 °C *LsGA2ox1* is more highly expressed in *L. sativa* Sal compared to *L. serriola* UC seeds. This is consistent with the finding that thermoinhibition causes elevated seed ABA contents, and that ABA promotes GA2ox-mediated GA inactivation (Seo *et al.*, 2006; Zentella *et al.*, 2007). Taken together, high temperature effects during seed thermoinhibition are caused by direct and indirect (via ABA) alteration of late GA metabolism which is mainly achieved by inhibited *GA20ox* and *GA3ox* gene expression (Figure 9.6c; Argyris *et al.*, 2008; Toh *et al.*, 2008).

## 9.5 Gibberellin and biotic stress factors: allelochemical interference of gibberellin biosynthesis during seed germination

Several plant- and microbe-derived phytotoxins have been proposed to be allelochemicals that affect seed germination and seedling growth of surrounding 'target' plants through leaching into the rhizosphere (Inderjit and Duke, 2003; Weston and Duke, 2003; Weir *et al.*, 2004). Examples of 'donor' plants with allelopathic phytotoxic potential include the juglon-producing walnut tree, as well as many invasive plant species. In many cases the mode of action of these allelochemicals is not known and, only in a few cases for seeds, was interference with hormone metabolism in the 'target' plant demonstrated (Bogatek and Gniazdowska, 2007; Oracz *et al.*, 2012; Voegele *et al.*, 2012). In addition to plant roots, microbes in the rhizosphere can also release phytotoxic compounds which interfere with GA-mediated plant

growth. An example is the rhizobacterium *Bacillus subtilis* IJ-31, for which the culture extract, as well as its component hydrocinnamic acid (HCA) were shown to act as plant growth retardants for red pepper, ryegrass and *Arabidopsis* (Kim and Rhee, 2012). A dose-dependent inhibition effect of HCA on seedling root and shoot growth in *Arabidopsis*, as well as the down-regulation of a GA-inducible cell-wall remodelling gene by HCA or the culture extract was demonstrated. Most interestingly, Kim and Rhee (2012) showed using an *in vitro* enzyme activity assay with recombinant AtGA3ox1 protein that HCA and the *Bacillus subtilis* IJ-31 culture extract both inhibit GA3ox enzyme activity. The effects of these putative allelochemicals on seed germination has not been studied, but HCA has been shown to inhibit seedling growth in other species (Tang and Young, 1982; Williamson *et al.*, 1992; Chon *et al.*, 2002).

Myrigalone A (MyA), 3-(1-oxo-3-phenylpropyl)-1,1,5-trimethylcyclohexane-2,4,6-trione (Figure 9.4e), a phytotoxin produced by *Myrica gale* L. ('sweet gale', 'bog myrtle', Myricaceae) has been demonstrated to inhibit seed germination and seedling growth of 'target' species (Popovici *et al.*, 2011; Oracz *et al.*, 2012; Voegele *et al.*, 2012). *M. gale* is a deciduous shrub native to Northern and Western Europe and North America, adapted to flood-prone habitats (Skene *et al.*, 2000). It grows in acidic peat bogs and at the intertidal zone of lakes and rivers that are often flooded by frequent rise and fall in water level. *M. gale* fruits and leaves secrete resin droplets containing essential oils (Figure 9.4e). Fruit and leaf exudates of *M. gale* exhibit phytotoxic activity on seedling growth of invasive knotweed (*Fallopia x bohemica*) and other species (Popovici *et al.*, 2011) and inhibit the seed germination of *L. sativum* (Oracz *et al.*, 2012; Voegele *et al.*, 2012). *M. gale* fruit exudates contain rare flavonoids, with MyA being the major C-methylated dihydrochalcone (Anthonsen *et al.*, 1971; Mathiesen *et al.*, 1995; Popovici *et al.*, 2011). MyA inhibits shoot and root growth of etiolated eudicot (cress, mustard, knotweed) and monocot (sorghum) seedlings (Popovici *et al.*, 2011), and also inhibits the growth of cress seedlings in the light (Oracz *et al.*, 2012). MyA inhibits processes required for embryo elongation during seed germination, including endoreduplication and the formation of apoplastic reactive oxygen in the hypocotyl-radicle axis of *L. sativum* (Oracz *et al.*, 2012). This finding is in agreement with the known function of MyA as a radical scavenger (Mathiesen *et al.*, 1997). Several key weakening and growth processes during early and late seed germination of *L. sativum* were found to be targets for MyA (Voegele *et al.*, 2012): MyA enhanced testa permeability and water uptake (early germination phase), and also inhibited endosperm weakening and rupture, and embryo growth (late germination phase). The inhibitory effects of MyA on *L. sativum* seed were modulated by light conditions and ambient water potential, with the inhibition being stronger in darkness compared to the light (Figure 9.4f). The important point with regard to the topic of this article is that the inhibition of seed germination was mediated, at least in part, by interference of MyA with GA metabolism and signalling (Oracz *et al.*, 2012; Voegele *et al.*, 2012).

MyA specifically interferes with GA-regulated processes important for seed germination of *L. sativum*, inhibiting endosperm weakening, as well as embryo extension growth. As shown in Figure 9.4, MyA causes a *ca.* threefold decrease of the (bioactive) $GA_4$ contents in the RAD of seeds imbibed in continuous light or in darkness, but does not affect the $GA_4$ contents in the CAP (Oracz *et al.*, 2012; Voegele *et al.*, 2012). This suggests that the MyA inhibition of endosperm rupture is mediated, at least in part, by GA-promoted downstream mechanism(s) that affects the embryo growth potential. The MyA-mediated inhibition of $GA_4$ production in the RAD was accompanied by a 200-fold (light) or 400-fold (darkness) accumulation of its biosynthetic precursor $GA_9$ (Figure 9.4b), indicating a block of the GA3ox-catalysed conversion of $GA_9$ to $GA_4$. MyA inhibits GA 3β-hydroxylation in the RAD, but does not affect it in the CAP (the increased $GA_9$ contents in the CAP may be due to diffusion from the RAD), and does not affect the preceding GA 20-oxidase reactions (Oracz *et al.*, 2012).

In contrast to the inhibitory effects of MyA on the $GA_4$ contents in the RAD, it had no appreciable inhibitory effect on $GA_1$ in this tissue, but the $GA_1$ contents were very low compared to the $GA_4$ contents (Figure 9.4c). GA 3-oxidase activity is also required for the production of $GA_6$ and $GA_{13}$ detected in RAD and CAP tissues (Figure 9.4d). In the light these steps are inhibited by MyA treatment in the RAD, but in the CAP MyA treatment caused a *ca.* 20-fold reduction in $GA_6$, but did not affect the contents of $GA_{13}$ (Oracz *et al.*, 2012; Voegele *et al.*, 2012). In darkness these effects of MyA were reversed (Figure 9.4d), suggesting that light modulates the MyA inhibition of GA metabolism. These findings also show that the MyA-mediated inhibition of GA3ox is not a general effect on these enzymes, but has some specificity regarding RAD/CAP and substrate/product for which the molecular mechanisms remain unknown. Taken together, MyA acts as a GA3ox inhibitor in germinating *L. sativum* seeds with specificity for the catalytic step from $GA_9$ to bioactive $GA_4$ and this compromises proper endosperm weakening and embryo growth required for endosperm rupture.

GA signalling via the soluble GID1-type GA receptors mediates downstream processes that confer embryo extension growth and endosperm weakening (see Section 9.3). A GA-triggered negative feedback loop in the CAP and RAD of germinating *L. sativum* seeds was only evident for the *GID1ac* transcripts, but not for *GID1b*. The GID1b receptor proteins have a higher $GA_4$ binding affinity ($K_D$ *ca.* 30 nM) than the GID1ac receptor proteins ($K_D$ *ca.* 300 nM) (Nakajima *et al.*, 2006). If we therefore assume that GA signalling is mediated only by the GID1ac receptor proteins in the germinating seed, the low concentrations of bioactive $GA_4$ after MyA treatment would not allow optimal GA signalling. Taken together, these results support the view that GA signalling via both GID1-pathways is required for the endosperm rupture of Brassicaceae seeds, and that MyA acts by interfering with GA metabolism and signalling important for downstream cell-wall loosening mechanisms such as XTH/expansins and/or apoplastic ROS (Voegele *et al.*, 2011; Oracz *et al.*, 2012; Voegele *et al.*, 2012). MyA therefore acts as an inhibitor

on important GA-regulated key processes of seed germination and seedling establishment as targets. Voegele *et al.* (2012) speculate that MyA is a soil seed bank-destroying allelochemical that secures the persistence of *M. gale* in its flood-prone environment.

## 9.6 Conclusions and perspectives

Seeds are diverse in structure and in a typical mature angiosperm seed the embryo is encased by a living endosperm layer (including the cereal grain aleurone) and a dead testa (seed coat) (Linkies *et al.*, 2010). The recent progress using novel approaches has deepened our knowledge about the roles of GA during seed germination. Important progress was obtained in seeds by moving away from whole-seed hormone and transcript quantification to seed tissues and key compartments (RAD, CAP), which for GA metabolites requires detection in minute amounts of tissue (Seo *et al.*, 2011; Oracz *et al.*, 2012; Urbanova *et al.*, 2013). The early induction of GA biosynthesis genes in the RAD is in accordance with the hypothesis that an embryonic GA metabolite and/or bioactive GA itself diffuses early during imbibition to the CAP to make it competent (release of coat dormancy) for the subsequent endosperm weakening during the late germination phase. Thus, the interaction between the key seed compartments is crucial for the control of seed germination by GA. GA signalling in seeds is distinct from seedlings, with expression of the GID1 receptor being part of this distinct regulation. Molecular phylogenetic analysis revealed that members of the angiosperm GID1 receptor family cluster into three distinct groups: eudicot GID1ac, eudicot GID1b and monocot GID1 (Voegele *et al.*, 2011). In Brassicaceae seeds the GID1ac receptors may be more important for the release of coat dormancy and the promotion of germination compared to the GID1b receptors, but proper seed germination requires both the GID1ac and the GID1b signalling pathways. All three types of GA receptor bind DELLA repressor proteins to target them for degradation which is important to promote seed germination. Environmental and hormonal, as well as developmental cues feed into these different variants of GA-signalling.

Gibberellin metabolism and signalling in seeds are involved in integrating environmental cues to control the timing of germination. Germination is influenced by abiotic stress factors, such as supra-optimal temperature (heat), which inhibits germination (Argyris *et al.*, 2008) and induction of *GA20ox* gene expression during early seed imbibition. Biotic stress factors such as the allelochemical myrigalone A (MyA) inhibit GA 3-oxidase enzyme activity and thereby the production of bioactive GAs required for seed germination (Oracz *et al.*, 2012; Voegele *et al.*, 2012). MyA targets different key processes, including the GA-induced endosperm weakening. These findings reinforce the importance of GA metabolism and signalling in mediating diverse

seed-environment interactions, and more of these are yet to be discovered. The GA requirement for seed germination was instrumental in screens at the dawn of *Arabidopsis* mutant research (Koornneef and van der Veen, 1980, Koorneef *et al.*, 1985). Gibberellins are, however, not simple 'GO AHEAD' (GA) molecules, since there are distinct and specific GA-actions in key seed compartments and beyond GA and ABA there is a complex network of signalling molecules that interact to control the timing of seed germination (Linkies and Leubner-Metzger, 2012).

## Acknowledgements

Support for positions and the research of the authors on gibberellins in seeds is gratefully acknowledged. This support was by the Deutsche Forschungsgemeinschaft (DFG LE720/6 and DFG LE720/7) to GLM, by Palacký University (Olomouc, CZ) (PrF_2013_023) and the National Program of Sustainability of the Czech Ministry of Education (LO1204) to TU.

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