J Plant Growth Regul (2010) 29:91–105
DOI 10.1007/s00344-009-9117-4

Profile of Plant Hormones and their Metabolites in Germinated and Ungerminated Canola (*Brassica napus*) Seeds Imbibed at 8°C in either GA_{4+7}, ABA, or a Saline Solution

Wentao Zhang · Sheila D. S. Chiwocha · Russell Trischuk · Lawrence V. Gusta

Received: 15 February 2009 / Accepted: 25 June 2009 / Published online: 16 September 2009

Abstract Abscisic acid (ABA) and gibberellins (GAs) are two major phytohormones that regulate seed germination in response to internal and external factors. In this study we used HPLC-ESI/MS/MS to investigate hormone profiles in canola (*Brassica napus*) seeds that were 25, 50, and 75% germinated and their ungerminated counterparts imbibed at 8°C in either water, 25 μM GA_{4+7}, a 80 mM saline solution, or 50 μM ABA, respectively. During germination, ABA levels declined while GA_4 levels increased. Higher ABA levels appeared in ungerminated seeds compared to germinated seeds. GA_4 levels were lower in seeds imbibed in the saline solution compared to seeds imbibed in water. Ungerminated seeds imbibed in ABA had lower GA_4 levels compared to ungerminated seeds imbibed in water; however, the levels of GA_4 were similar for germinated seeds imbibed in either water or ABA. The ABA metabolites PA and DPA increased in seeds imbibed in either water, the saline solution, or ABA, but decreased in GA_{4+7}-imbibed seeds. In addition, ABA inhibited GA_4 accumulation, whereas GA had no effect on ABA accumulation but altered the ABA catabolism pathway. Information from our studies strongly supports the concept that the balance of ABA and GA is a major factor controlling germination.

Keywords Seed germination · Hormones · Metabolites · HPLC-ESI/MS/MS

W. Zhang
Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, Saskatchewan S7N-0X2, Canada

S. D. S. Chiwocha
ARC COE, Plant Energy Biology, Research School of Biological Science, Austrialian National University, ACT-0200 Canberra, Austrialia

R. Trischuk
Dow AgroSciences Canada Inc, 101-421 Downey Road, Saskatoon, Saskatchewan S7N-4L8, Canada

L. V. Gusta (✉)
Department of Plant Sciences, College of Agriculture and Bioresources, University of Saskatchewan, 51 Campus Drive, Room 4D66, Agriculture Building, Saskatoon, Saskatchewan S7N-5A8, Canada
e-mail: larry.gusta@usask.ca

Introduction

Seed germination is an important process in the life history of plants and its completion sets in motion the growth of the seedling (Millar and others 2006). Seed germination begins when a quiescent seed uptakes water and is completed with the elongation and emergence of the radicle in a turgor-driven process (Finch-Savage and Leubner-Metzger 2006). Germination is a very complex physiological process that is controlled by a range of developmental and external cues. Genetic and physiological studies have shown the important role played by plant hormones in regulating seed germination (Karssen and others 1989; Jacobsen and others 2002; Koornneef and others 2002).

Studies on the genetic control of seed germination have focused mainly on hormone biosynthesis and hormone-responsive mutants. Through these studies, abscisic acid (ABA) and gibberellins (GAs) have been demonstrated to play important roles in the control of seed dormancy and germination. For example, in gibberellin-deficient *Arabidopsis* and tomato mutants, the full germination response required the application of GA to the medium (Koornneef and Van Der Veen 1980; Groot and Karssen 1987). ABA-deficient (*aba*) and ABA-insensitive (*abi*)

mutants of *Arabidopsis* exhibit reduced seed dormancy (Koornneef and others 1982; Karssen and others 1983; Koornneef and others 1984; Debeaujon and Koornneef 2000; Jacobsen and others 2002), whereas exogenous ABA or overproduction of ABA delayed seed germination or enhanced seed dormancy (Frey and others 1999; Thompson and others 2000; Lindgren and others 2003; Nambara and Marion-Poll 2003). Previous studies proposed that ABA induces and maintains seed dormancy (Nambara and Marion-Poll 2003; Kucera and others 2005), whereas GA, which is antagonistic to the effect of ABA, releases seed dormancy and promotes seed germination (Debeaujon and Koornneef 2000; Ogawa and others 2003; Yamauchi and others 2004; Kucera and others 2005). The stimulatory role of GAs on small-seeded plants such as tomato and *Arabidopsis* may be explained by at least two different mechanisms. First, GAs induce certain hydrolytic enzymes to overcome the mechanical resistance imposed by the endosperm and seed coat (Debeaujon and Koornneef 2000). For example, several cell wall-loosening genes that encode β-1,3-glucanase and endo-β-mannanase are GA-inducible and are consistently associated with germination (Nonogaki and others 2000; Wu and others 2001; Leubner-Metzger 2002; Koornneef and others 2002; Wu and Bradford 2003). Second, GAs increase the growth potential of the embryo, as indicated in *Arabidopsis* (Karssen and Lacka 1986; Debeaujon and Koornneef 2000).

Previous studies indicated that GA-mediated developmental processes are regulated in part by changing the cellular concentration of bioactive GAs (Yamauchi and others 2004). In barley and *Arabidopsis* seeds, GA increases during germination (Karssen and others 1989; Ogawa and others 2003; Yamauchi and others 2004). Recent studies have shown that breaking dormancy by after-ripening, stratification, dark, and smoke is strongly correlated with a decrease of ABA in seeds (Gubler and others 2005). In addition, dormant cultivars of wheat and barley contained more ABA than nondormant cultivars (Goldbach and Michael 1976; Walker-Simmons and Sesing 1990). However, some studies suggested that seed germination is determined by the concentration of ABA in imbibed seeds and not by the concentration in dry seeds (Millar and others 2006). For example, in dormant and nondormant *Arabidopsis* seeds or embryos of barley, germination ability was highly correlated with the changing pattern of ABA upon imbibition (Ali-Rachedi and others 2004; Millar and others 2006). Hormone levels were shown to be strongly influenced by various endogenous and external signals (Ogawa and others 2003; Ali-Rachedi and others 2004; Yamauchi and others 2004; Millar and others 2006). Besides hormone levels, hormone sensitivity also plays an important role in seed germination. In *Arabidopsis*, seed germination in response to light and low-temperature stimuli was identified to be due to enhanced GA sensitivity, not the amount of GA (Derkx and others 1994).

The endogenous level of a given plant hormone is controlled by biosynthesis and catabolism. De novo GA and ABA biosynthesis during imbibition was demonstrated by the following observations: an inhibitor of GA biosynthesis, paclobutrazol, inhibits seed germination, which contrasts with the enhanced effects of fluridone or norflurazon, which are ABA biosynthesis inhibitors (Le Page-Degivry and Garello 1992; Debeaujon and Koornneef 2000). Molecular studies indicated that two genes (*NCED6* and *9*) belonging to the *Arabidopsis* 9-cis-epoxycarotenoid dioxygenase (*AtNCED*) gene family are the major genes responsible for ABA synthesis during *Arabidopsis* seed development and germination (Tan and others 2003; Lefebvre and others 2006). At the same time, ABA 8′-hydroxylase, the key enzyme in ABA catabolism, was found to be indispensable for proper control of seed dormancy and germination (Millar and others 2006). Gene expression studies revealed that several GA biosynthesis genes such as *ent-kaurene oxidase* (*AtkO1*), *GA 20-oxidase*, *GA 3-oxidase1* (*AtGA30x1*), and *GA 3-oxidase2* (*AtGA3ox2*) are upregulated during seed imbibition and are involved in the GA control of seed germination (Ogawa and others 2003; Pérez-Flores and others 2003; Yamauchi and others 2004). *Gibberellin 2-oxidase* (*AtGA2ox2*) gene is responsible for the deactivation of bioactive GAs (Yamauchi and others 2007). The precise control of the expression of these genes indicates that fine-tuning of hormone levels is an important signal for plant responses to the environmental factors. Therefore, studying the hormone profiles and their metabolites is an invaluable tool for investigating the role played by plant hormones during seed germination.

Low temperatures and salinity are considered important stress factors limiting seed germination, emergence, and stand establishment, particularly for canola (*Brassica napus*), a small-seeded crop. Although information on the roles of hormones in the process of seed germination has greatly increased, knowledge of their roles in seeds subjected to low temperatures and abiotic stressful conditions is minimal. Because of the long cold winter in western Canada, the soil is often frozen to a depth of 1 m. Because of the short growing season, early seeding in May is common when soil temperatures are generally lower than 10°C. Therefore, in this study the effects of low temperatures and salinity on hormone profiles were studied at 8°C in both germinated and ungerminated canola seeds.

In this study we used high-performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC-ESI/MS/MS) to profile ABA, ABA metabolites, gibberellins, auxins, and cytokinins in canola seeds (cv. black seed line, N89-53) imbibed at 8°C in either

water, 25 μM GA_{4+7}, an 80 mM buffered saline solution of K_2HPO_4-K_2HPO_4 (pH 7.0), or 50 μM ABA when 25, 50, and 75% of the seeds were considered to have germinated and also the counterparts of the ungerminated seeds (75, 50, and 25%).

Materials and Methods

Plant Material and Seed Germination

Brassica napus seeds, a black seed genotype N89-53 obtained from Dr. G. Rakow, Agriculture and Agri-Food Canada (Saskatoon, SK, Canada), were imbibed at 8°C in either water, 25 μM GA_{4+7}, a buffered saline solution (80 mM K_2HPO_4-KH_2PO_4, pH 7.0), or 50 μM S(+) ABA in the absence of light. All of the experiments were replicated four times in Petri dishes with 100 seeds per dish imbibed on filter paper with 5 ml of the above solutions. Both germinated (25, 50, and 75%) and ungerminated seeds (75, 50, and 25%) were collected for hormonal analysis.

Extraction of Plant Hormones and Metabolites

The extraction procedure was as described in Chiwocha and others (2003), except that 80% isopropanol acidified with 1% glacial acetic acid was used as the extraction solution. The extraction for each sample was replicated three times.

Analysis of Endogenous Plant Hormones and Metabolites by HPLC-ESI/MS/MS

The following plant hormones and their metabolites were profiled for each collection: (1) ABA and metabolites: ABA, phaseic acid (PA), dihydrophaseic acid (DPA), 7′-hydroxy ABA (7′-OH ABA), neo-phaseic acid (neo-PA), and ABA glucose ester (ABAGE); (2) gibberellins (GA_1, GA_3, GA_4, and GA_7; (3) auxins: indole-3-acetic acid (IAA) and indole-3-aspartate (IAAsp); and (4) cytokinins: isopentenyladenine (2iP), isopentenyladenosine (IPA), zeatin (Z), zeatin riboside (ZR), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), and zeatin-O-glucoside (Z-O-Glu). The retention times, precursor-to-product-ion transitions, and the procedure used for quantification of endogenous plant hormones and metabolites using the deuterium-labeled analog of each compound as its internal standard were as described previously in Chiwocha and others (2003). The precursor-to-product-ion transitions used to monitor and quantify each compound and their internal standard are shown as the following: ABA (*m/z* 263 > 153), d_4-ABA (*m/z* 267 > 156), PA (*m/z* 279 > 139), d_3-PA (*m/z* 282 > 142), DPA (*m/z* 281 > 171), d_3-DPA (*m/z* 284 > 174), 7′-OH-ABA (*m/z* 279 > 151), d_4-7′-OH-ABA (*m/z* 283 > 154), ABA-GE (*m/z* 425 > 263), d_5-ABA-GE (*m/z* 430 > 268); GA_1 (*m/z* 347 > 273), d_2-GA_1 (*m/z* 349 > 275), GA_3 (*m/z* 345 > 221), d_2-GA_1 (*m/z* 349 > 275), GA_4 (*m/z* 331 > 213), d_2-GA_4 (*m/z* 333 > 215), GA_7 (*m/z* 339 > 223), d_2-GA_4 (*m/z* 333 > 215); IAA (*m/z* 174 > 130), d_5-IAA (179 > 135), IAAsp (*m/z* 289 > 132), d_5-IAA (*m/z* 179 > 1350; 2ip (m/z 204 > 136), d_6-2iP (210 > 137), IPA (m/z 336 > 204), d_6-IPA (m/z 342 > 210), Z (*m/z* 220 > 136), d_3-DHZ (*m/z* 225 > 136), ZR (*m/z* 352 > 220), d_3-DHZR (*m/z* 337 > 225), DHZ (*m/z* 222 > 136), d_3-DHZ (*m/z* 225 > 136), DHZR (*m/z* 353 > 222), d_3-DHZR (*m/z* 357 > 223), Z-O-Glu (*m/z* 382 > 220), and d_5-Z-O-Glu (*m/z* 387 > 225). d_2-GA_1, d_2-GA_4, d_5-IAA, d_3-DHZ, and d_3-DHZR were also found to be appropriate internal standards for GA_3, GA_7, IAAsp, Z, and ZR, respectively. Each sample was injected and analyzed in triplicate by HPLC-ESI/MS/MS.

Results

Germination Response of *Brassica napus* Seeds at 8°C Employing Various Incubation Media

The germination time course of *Brassica napus* seeds imbibed at 8°C in either water, 25 μM GA_{4+7} (GA), an 80 mM saline solution, or 50 μM ABA in the absence of light is shown in Fig. 1. Compared with seeds imbibed in water, GA stimulated seed germination, whereas the saline solution or ABA inhibited seed germination.

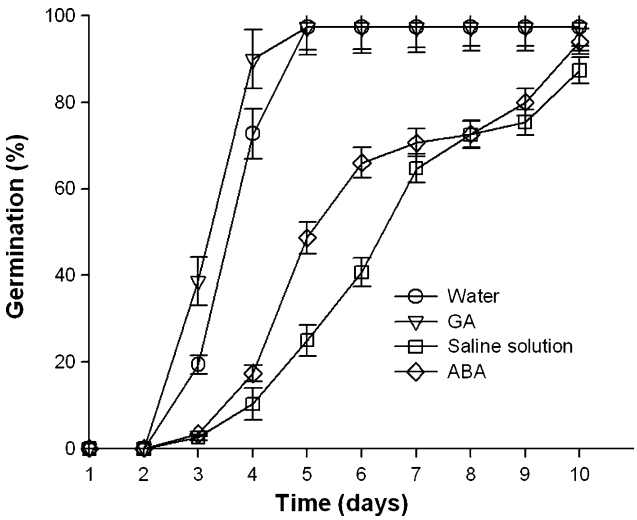

Fig. 1 Germination profiles of *Brassica napus* (N89-53) imbibed at 8°C in either water, 25 μM GA_{4+7}, a 80 mM saline solution, or 50 μM ABA in the absence of light. Germination was scored as radicle emergence. Values are means ± SE of four replicates

Hormone Profiles During Germination

Both germinated and ungerminated seeds imbibed at 8°C in either water, GA_{4+7}, the saline solution, or ABA were collected at 25, 50, and 75% germination for hormonal analysis. The differences among the endogenous hormones and their metabolites at the different stages of germination are presented.

ABA

Changes in the levels of ABA at 25, 50, and 75% germination and their ungerminated counterparts are shown in Fig. 2. It was not possible to determine ABA levels in seeds imbibed in exogenous ABA; however, it was possible to measure the ABA metabolites that are discussed later (Figs. 3 and 4). The level of ABA in dry seeds was 70 ng g^{-1} dry weight (DW), which in all cases decreased to less than 26 ng g^{-1} DW in germinated seed (Fig. 2). A greater decrease was observed in seeds imbibed in the saline solution (20 ng g^{-1} DW or less) compared with the 26 ng g^{-1} DW for GA-imbibed seeds (Fig. 2). In all the treatments, there was little or no change in the levels of ABA as germination proceeded. ABA levels also decreased in the ungerminated seeds but not to the extent observed in the germinated seeds. For example, for 75% of ungerminated seeds, the concentration of ABA was approximately 35 ng g^{-1} DW compared with 26 ng g^{-1} DW for 25% germinated seeds imbibed in water (Fig. 2). Surprisingly, the ABA concentration in 75% ungerminated GA-imbibed seeds was similar to the level observed in seeds imbibed in the saline solution. In all the treatments, the level of ABA was higher in the 25% ungerminated seed, which was the slowest to germinate. The 50% stage is considered to have the highest rate of germination and this is reflected by the levels of ABA.

ABA Metabolites

The main ABA metabolite stored in dry seeds was ABA-GE, followed by DPA which was nearly half of the concentration as ABA-GE, then 7′ OH-ABA, and finally PA (Figs. 3 and 4). During imbibition, ABA-GE remained relatively constant over time in both the germinated and ungerminated seeds imbibed (Fig. 3a). In contrast, there was an approximately 40% increase in ABA-GE for seeds imbibed in GA_{4+7}, suggesting that it is the major ABA catabolite (Fig. 3b). DPA increased in seeds imbibed in water; however, it increased more in germinated seeds compared with ungerminated seeds (Fig. 3a). In the presence of GA_{4+7}, DPA decreased in ungerminated seeds and was not detectable in germinated seeds (Fig. 3b), suggesting a different pathway of ABA catabolism. There was a slight decrease in 7′-OH-ABA in both germinated and ungerminated seeds imbibed in either water or GA, whereas PA increased slightly (Fig. 3).

Changes in ABA-GE in saline solution-treated seeds were similar to those observed for both germinated and ungerminated seeds imbibed in water (Fig. 4a). The increase in DPA for saline solution-treated ungerminated seeds was similar to ungerminated seeds imbibed in water; however, there was a 35% decrease in the germinated seeds compared with a 38% increase in the water-imbibed geminated seeds (Figs. 3a and 4a). The concentration of 7′-OH-ABA in the saline solution-treated seeds was similar to the germinated water- and GA-imbibed seeds and the water-imbibed ungerminated seeds. In contrast to the water- and GA-treated seeds and the saline solution-treated

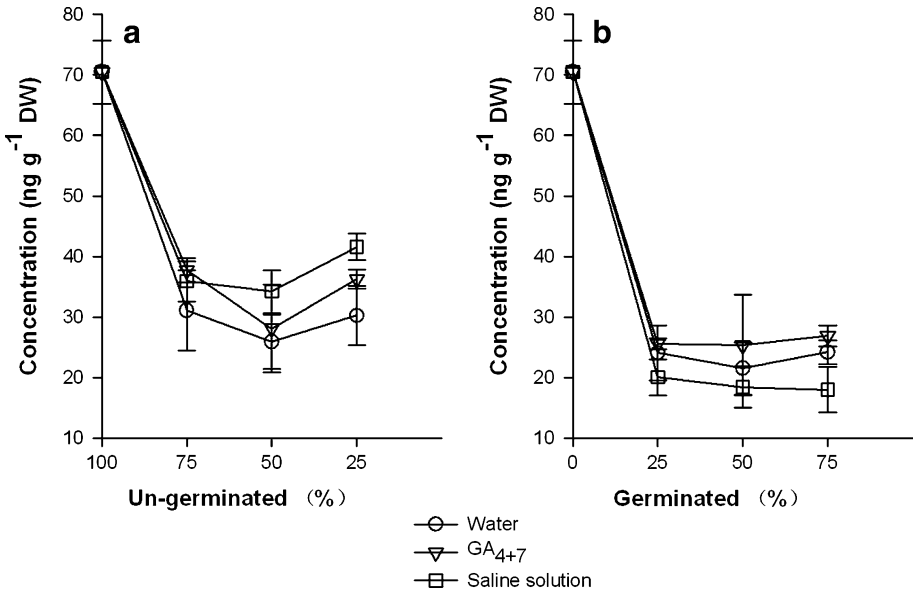

Fig. 2 Changes in ABA in both germinated and ungerminated seeds imbibed at 8°C in either water, a saline solution, or GA_{4+7} in the absence of light. Seeds were collected at 0, 25, 50, and 75% germination and also their ungerminated counterparts at the same intervals. **a** Ungerminated. **b** Germinated. Values are means ± SE of three replicates

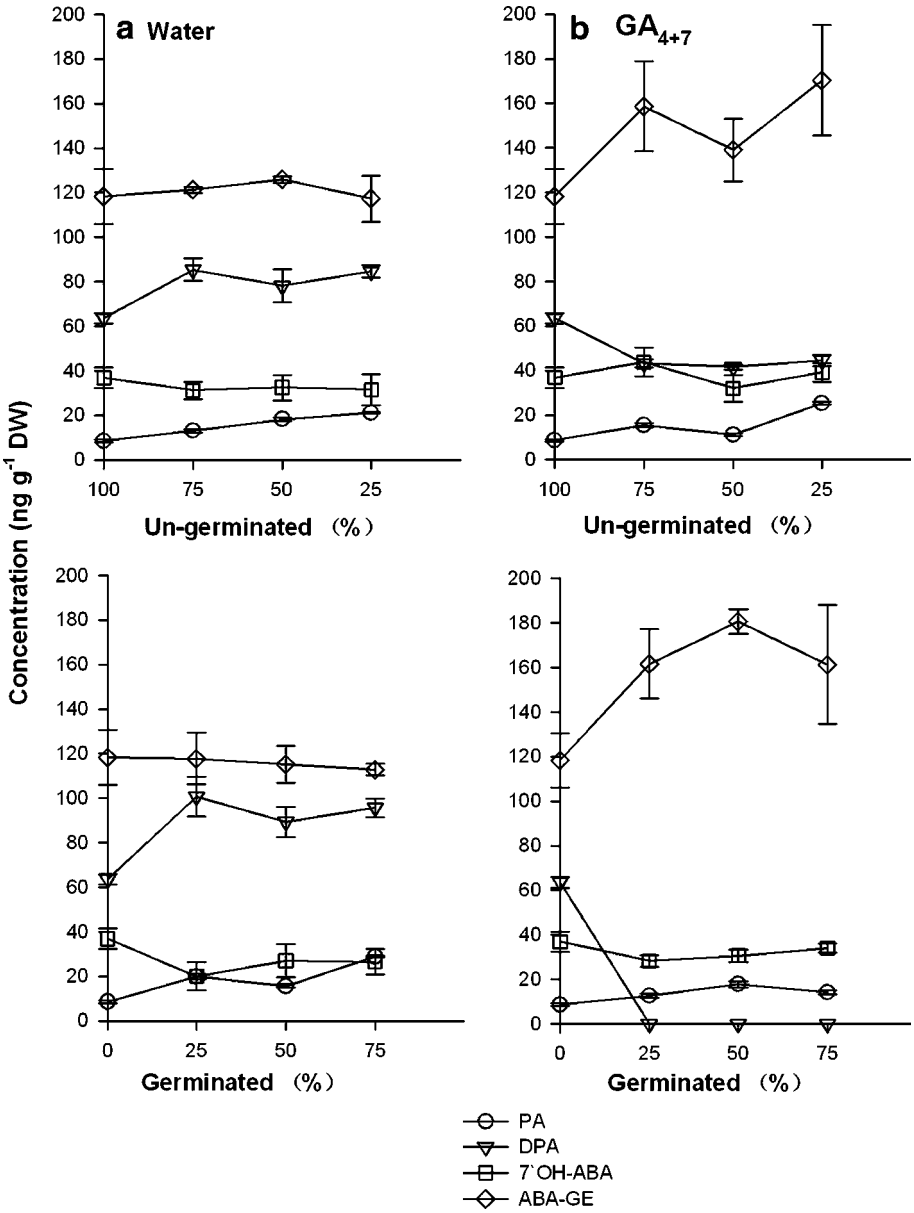

Fig. 3 Changes in ABA metabolites in both germinated and ungerminated seeds imbibed at 8°C in either water or GA_{4+7} in the absence of light. Seeds were collected at 0, 25, 50, and 75% germination and also their ungerminated counterparts at the same intervals. **a** Water. **b** GA_{4+7}. Values are means ± SE of three replicates

ungerminated seeds, there was a major increase in PA in the germinated saline solution-treated seeds (Fig. 4a). In one of the three ABA catabolytic pathways, ABA is catabolized first to PA and then DPA (Harrison and Walton 1975). It appears that salinity affects the conversion of PA to DPA. Although ABA-GE was the major ABA catabolite in germinated GA-treated seeds, DPA was the major catabolite in germinated ABA-treated seeds (Figs. 3b and 4b). DPA increased from 120 ng g^{-1} DW in dry seeds to 155 ng g^{-1} DW in ABA-imbibed seeds at 25% germination, and increased to greater than 400 ng g^{-1} DW in seeds at 50 and 75% germination. ABA-GE also increased in both germinated and ungerminated seeds imbibed in ABA; however, its increase was not as large as observed for DPA (Fig. 4b).

Gibberellins

GA_3 was not detected in dry seeds (Fig. 5), but at 25% germination, GA_3 increased to approximately 50 ng g^{-1} DW in seeds imbibed in water (Fig. 5). At 50% germination, GA_3 decreased to approximately 35 ng g^{-1} DW and then increased to 60 ng g^{-1} DW at 75% germination (Fig. 5). There was a significant increase in GA_3 in the 75% ungerminated seeds imbibed in water, but then GA_3 decreased from 27 ng g^{-1} DW and remained constant at 15 ng g^{-1} DW in the 50 and 25 % ungerminated seeds (Fig. 5). Trace amounts of GA_4 (4 ng g^{-1} DW) were detected in dry seeds, which increased to 18 ng g^{-1} DW when 25% of the water-imbibed seeds germinated and remained relatively constant thereafter (Fig. 5). The level

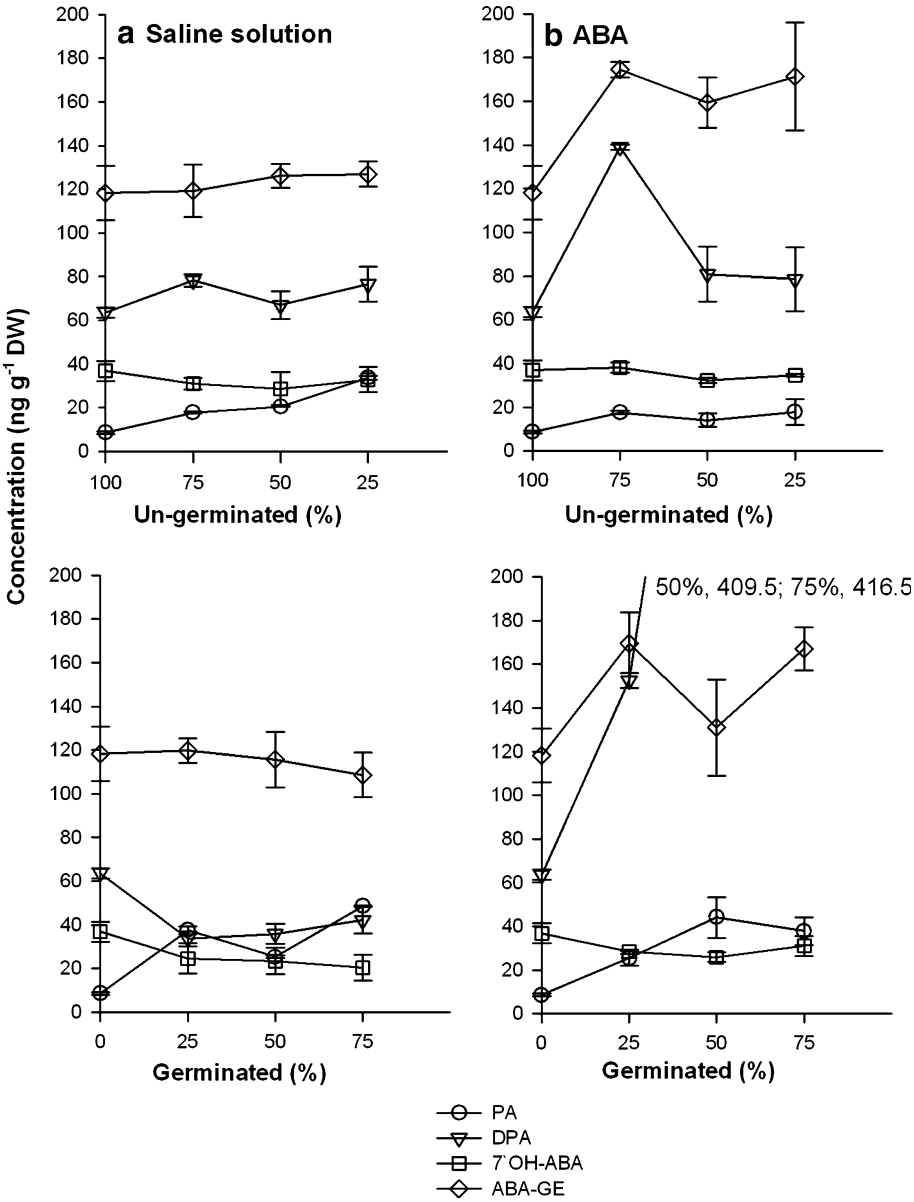

Fig. 4 Changes in ABA metabolites in both germinated and ungerminated seeds imbibed at 8°C in either the saline solution or ABA in the absence of light. Seeds were collected at 0, 25, 50, and 75% germination and also their ungerminated counterparts at the same intervals. **a** Saline solution. **b** ABA. Values are means ± SE of three replicates

of GA_4 in ungerminated seeds was similar to what was observed in germinated seeds imbibed in water at all stages of germination (Fig. 5). In contrast to the increase in GA_3 for seeds imbibed in water, there was no increase in GA_3 in seeds imbibed in either GA_{4+7} (data not shown), the saline solution, or ABA (Fig. 6). The increase in GA_4 was less in seeds imbibed in the saline solution compared with seeds imbibed in water (Fig. 6a). There was a twofold decrease in GA_4 in seeds after 75% germination. A similar pattern was observed in ungerminated seeds as was observed for seeds imbibed in water, except the increase in GA_4 was approximately 47% less (Fig. 6a). In ABA-treated germinated seeds the increase in GA_4 was similar to what was observed in water-treated germinated seeds (Fig. 6b). GA_4 increased in ABA-imbibed 75% ungerminated seeds, almost to the level observed in water-treated ungerminated seeds (Fig. 6b). Thereafter, GA_4 decreased and decreased 50% in 25% ungerminated seeds (Fig. 6b). GA_1 and GA_7 were not detected in our study.

Auxins

There was a significant increase in IAA at all stages of germination in water; however, IAA increased more in ungerminated seeds (Fig. 7a). IAAsp levels remained constant at all stages (Fig. 7a). In contrast to water-imbibed seeds, there were little or no changes in either IAA or IAAsp in seeds imbibed in either GA_{4+7}, the saline solution, or ABA (Figs. 7b and 8).

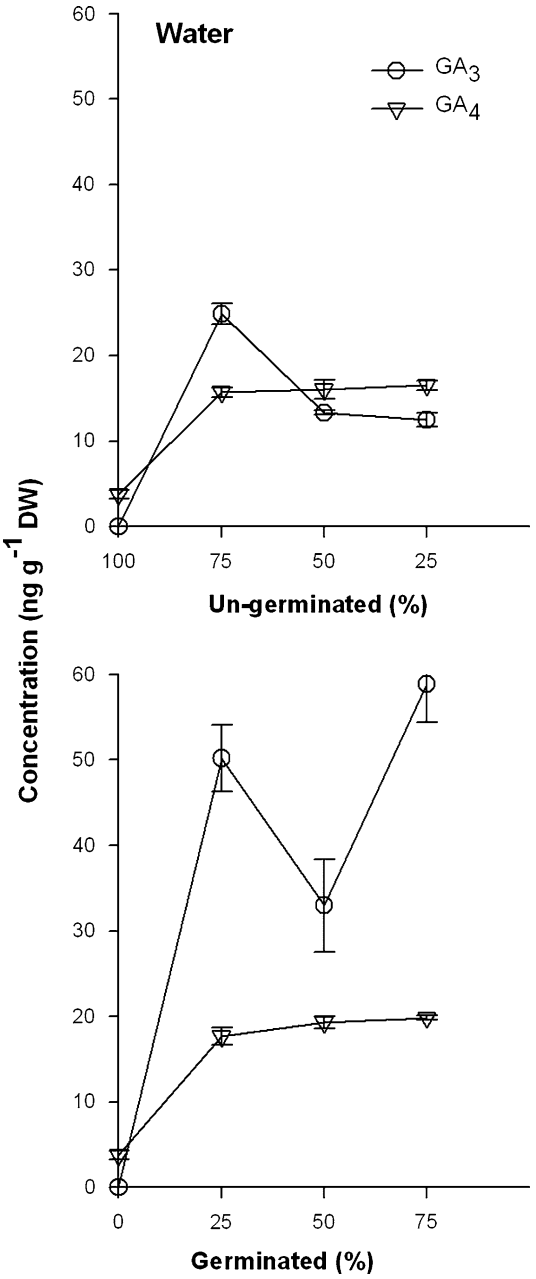

Fig. 5 Changes in gibberellins in both germinated and ungerminated seeds imbibed at 8°C in water in the absence of light. Seeds were collected at 0, 25, 50, and 75% germination and also their ungerminated counterparts at the same intervals. Values are means ± SE of three replicates

Cytokinins

ZR, iPA, and DhZR were detected in either germinated or ungerminated seeds in all treatments except seeds imbibed in ABA (Figs. 9 and 10). DhZR increased markedly in seeds imbibed in water but was not detected in seeds imbibed in GA_{4+7} (Fig. 9). Compared with dry seeds, iPA increased in seeds imbibed in either water or GA_{4+7} (Fig. 9). The largest increase was observed in seeds imbibed in water at 75% of germination. For seeds imbibed in the saline solution, ZR remained at the same level as in the dry seeds (Fig. 10). The levels of DhZR increased in both germinated and ungerminated seeds imbibed in the saline solution; however, iPA was not detected in either (Fig. 10).

Discussion

Compared to what occurs with water, seeds germinate slightly faster in the presence of GA_{4+7} and slower when imbibed in either the saline solution or ABA (Fig. 1). This different germination pattern is thought to be controlled by endogenous hormones such as GA or ABA. To elucidate how hormones control germination, we profiled changes in ABA and its metabolites, gibberellins (GA_3 and GA_4), auxins (IAA and IAAsp), and cytokinins (ZR, dhZR, and iPA) in both germinated and ungerminated seeds at different stages of germination. In addition, we added exogenous GA_{4+7}, ABA, or a saline solution to the seeds to determine what effect they had on the hormones. Profiles of ABA and its metabolites for seeds imbibed in GA_{4+7} and profiles of gibberellins for seeds imbibed in ABA are discussed in detail in the section on interactions between GA and ABA.

ABA and its Metabolism

ABA is a negative regulator in the control of seed germination (Finch-Savage and Leubner-Metzger 2006). ABA levels in seeds are regulated by anabolism and catabolism (Okamoto and others 2006). In our study, ABA levels decreased in all cases upon imbibition, irrespective of the treatments (Fig. 2), which is consistent with the pattern of ABA changes observed in *Arabidopsis* (Ali-Rachedi and others 2004) and barley (Millar and others 2006). Moreover, our results reveal that ungerminated seeds contained higher ABA levels than germinated seeds in all the treatments. These results indicate that a decline in ABA is required for the initiation of germination and there is a threshold level controlling seed germination. Seeds imbibed in the saline solution germinated slower than seeds imbibed in water (Fig. 1). Compared with ungerminated seeds imbibed in water, ABA levels were higher in ungerminated seeds imbibed in the saline solution; however, they were slightly lower in germinated seeds imbibed in the saline solution. This suggests that the delay in germination for seeds imbibed in the saline solution may be a function of the time required for catabolism of ABA at 8°C below the threshold level. This concept is supported by previous studies that demonstrated that there is a threshold level for ABA inhibition of germination (Millar and others 2006).

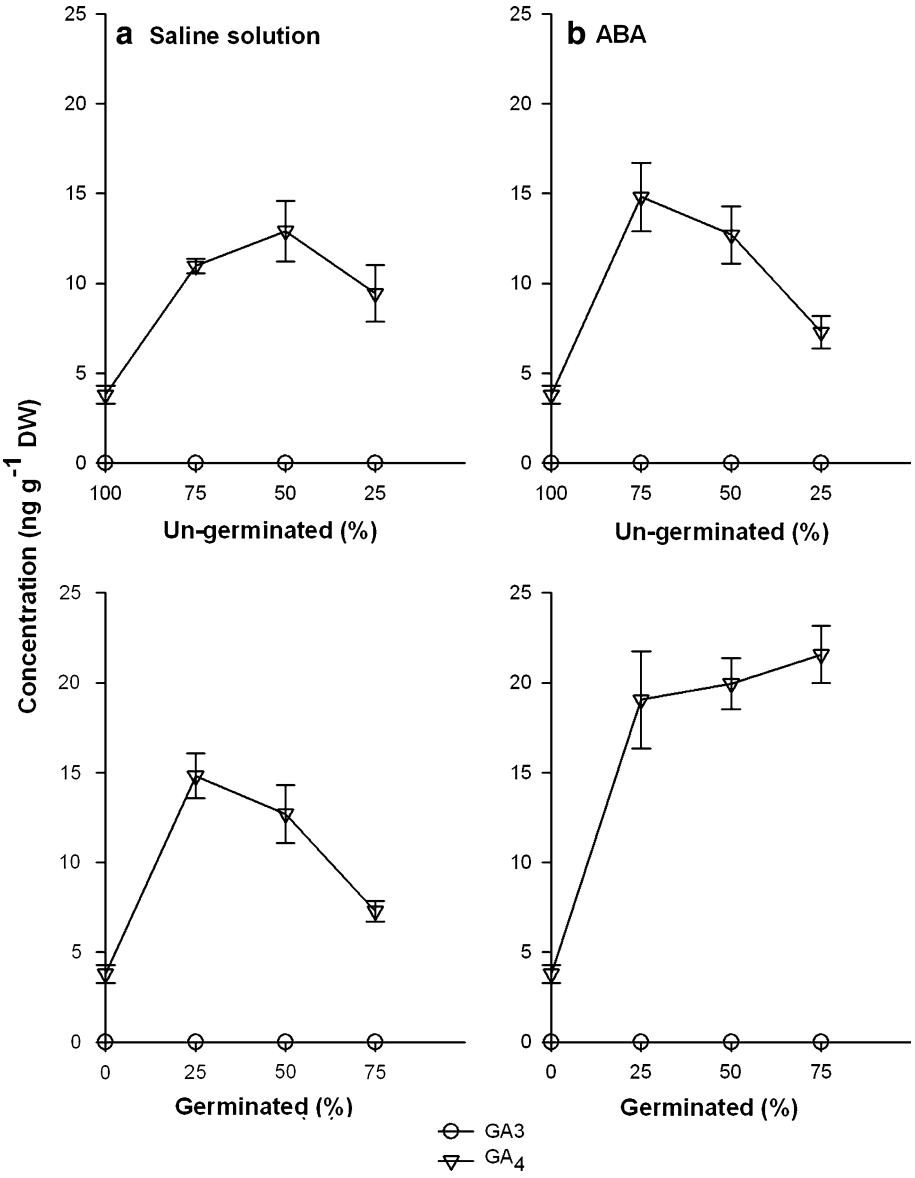

Fig. 6 Changes in gibberellins in both germinated and ungerminated seeds imbibed at 8°C in either the saline solution or ABA in the absence of light. Seeds were collected at 0, 25, 50, and 75% germination and also their ungerminated counterparts at the same intervals. **a** Saline solution. **b** ABA. Values are means ± SE of three replicates

Higher ABA levels observed in ungerminated seeds imbibed in the saline solution may be the result of induced ABA de novo synthesis or the delayed rate of ABA catabolism. Previous studies in lettuce (Yoshioka and others 1998; Gonai and others 2004), and tomato (Fellner and Sawhney 2001) demonstrated that delayed seed germination under stressful conditions is partially due to induced ABA de novo synthesis. However, fluridone, an ABA inhibitor (Yoshioka and others 1998), had no effect on the germination of seeds imbibed in the saline solution as described in the current study (Zhang and Gusta, unpublished data). The higher ABA levels observed in our study may be attributed to the rate of ABA catabolism, which is inhibited by the saline solution rather than de novo ABA synthesis. Previous studies in either *Arabidopsis* (Ali-Rachedi and others 2004), lettuce (Toyomasu and others 1994), or barley (Millar and others 2006) demonstrated a correlation between ABA levels in imbibed seeds and seed germinability. In our study, the lowest level of ABA was found in 50% ungerminated seeds followed by an increase in 25% ungerminated seeds. Seeds at 50% germination have the highest germination rate, whereas seeds at 75% germination have the slowest germination rate. These results provide evidence to support the concept that endogenous ABA concentration is associated with seed germinability.

Previous studies have established that ABA is catabolized through two major oxidation pathways: 8′ hydroxylation to PA and then to DPA and 7′ hydroxylation to 7′-OH-ABA (Uknes and Ho 1984; Cutler and Krochko 1999) or a conjugation pathway to ABA-GE (Zhou and others 2004). In *Arabidopsis*, molecular studies with *CYP707A1* and *CYP707A2* genes, which encode two key

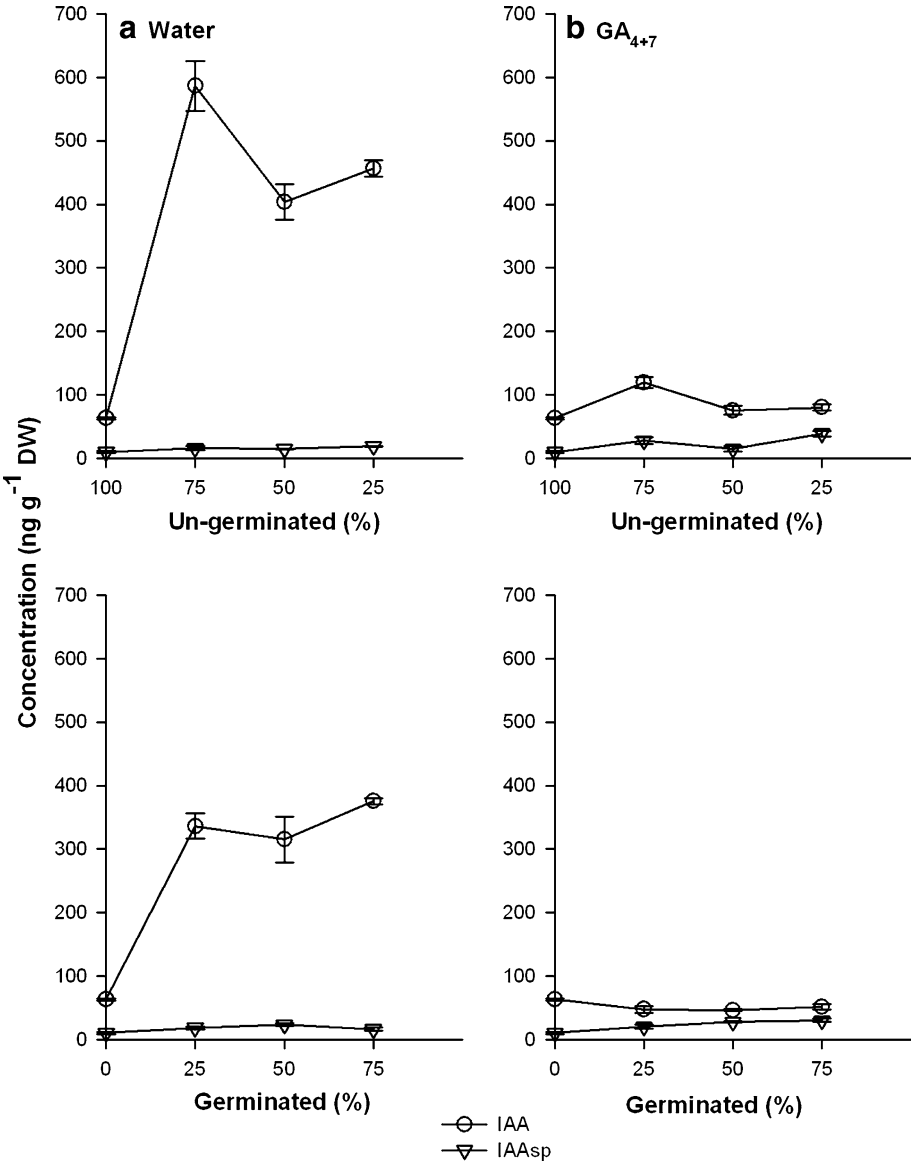

Fig. 7 Changes in auxins in both germinated and ungerminated seeds imbibed at 8°C in either water or GA_{4+7} in the absence of light. Seeds were collected at 0, 25, 50, and 75% germination and also their ungerminated counterparts at the same intervals. **a** Water. **b** GA_{4+7}. Values are means ± SE of three replicates

enzymes in the ABA 8′-hydroxylase pathway, revealed that the 8′ hydroxylation pathway is the major pathway involved in ABA-controlled germination (Kushiro and others 2004; Millar and others 2006; Okamoto and others 2006). In our study, both PA and DPA increased in seeds imbibed in water, as expected with the observed reduction in ABA (Fig. 3); however, 7′-OH-ABA levels did not vary significantly in ungerminated seeds and slightly decreased in germinated seeds (Fig. 3). ABA-GE also remained relatively constant in seeds imbibed in water, although it was the major ABA catabolite in dry seeds. These results indicate that the 8′ hydroxylation is the preferred pathway for ABA catabolism in canola seeds imbibed in water at 8°C, which is consistent with previous studies. For germinated seeds imbibed in the saline solution, PA was higher compared to water-imbibed germinated seeds, whereas DPA decreased, which is in contrast to the increase observed in germinated seeds imbibed in water (Fig. 4). These results indicate that the conversion of PA to DPA in seeds is affected by the saline solution. For seeds imbibed in ABA, DPA was significantly enhanced compared with water-imbibed seeds. This observation is consistent with the concept that ABA itself can activate its 8′ hydroxylation catabolic pathway (Uknes and Ho 1984; Cutler and Krochko 1999; Qin and Zeevaart 2002). In our study we found that ABA-GE was also greatly enhanced in seeds imbibed in ABA. It appears that the ABA-GE conjugation pathway is also activated via exogenous ABA. This is different from previous studies in plants that have shown that only the 8′ hydroxylation is activated (Uknes and Ho 1984; Cutler and Krochko 1999; Qin and Zeevaart 2002).

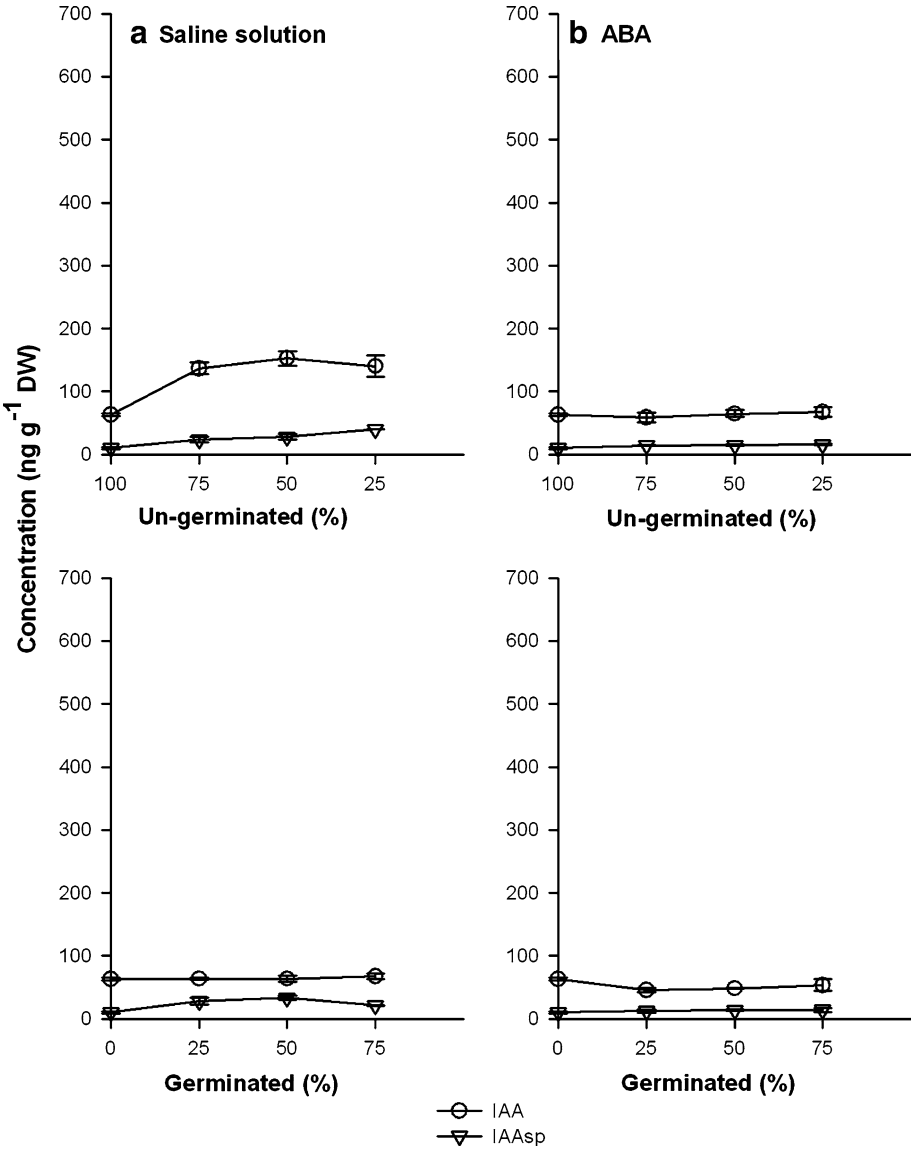

Fig. 8 Changes in auxins in both germinated and ungerminated seeds imbibed at 8°C in either the saline solution or ABA in the absence of light. Seeds were collected at 0, 25, 50, and 75% germination and also their ungerminated counterparts at the same intervals. **a** Saline solution. **b** ABA. Values are means ± SE of three replicates

Gibberellins

GAs have been shown to be required for germination from studies on GA-deficient mutants (Koornneef and Van Der Veen 1980; Groot and Karssen 1987) and GA biosynthesis inhibitors (Karssen and others 1989; Nambara and others 1991). GA_4, a bioactive gibberellin, increases in imbibed seeds, indicating that GA_4 is essential for seed germination. GA_4 may be a major bioactive gibberellin for canola seed germination at 8°C. This finding is consistent with studies on *Arabidopsis* which also demonstrated the essential role of GA_4 in seed germination (Ogawa and others 2003; Yamauchi and others 2004). GA_4 was lower in seeds imbibed in the saline solution compared with that in seeds imbibed in water. After 75% of the seeds germinated in the saline solution, the 25% ungerminated seeds had the lowest GA_4 (Fig. 6). These results indicate that salinity-delayed seed germination is partly induced by its inhibitory effect on GA_4 accumulation. In combination with the ABA profiles for seeds imbibed in the saline solution, we propose that the saline solution inhibits seed germination by reducing ABA catabolism as well as GA accumulation. In addition to GA_4, GA_3, another bioactive gibberellin, was found to accumulate only in seeds imbibed in water but not in the saline solution or ABA. This result indicates that the GA_3 biosynthetic pathway was also activated in seeds imbibed in water at 8°C; however, the saline solution and ABA inhibited this step. Until now, a large number of GAs (approximately 126) have been identified in higher plants, fungi, and bacteria (Hedden and Phillips 2000). The GAs vary in activity; for example, previous studies have shown that GA_1, GA_3, GA_4, and GA_7 all stimulate *Arabidopsis* seed germination, although the extent of the activity varies (Derkx and others 1994). However, Ogawa and others

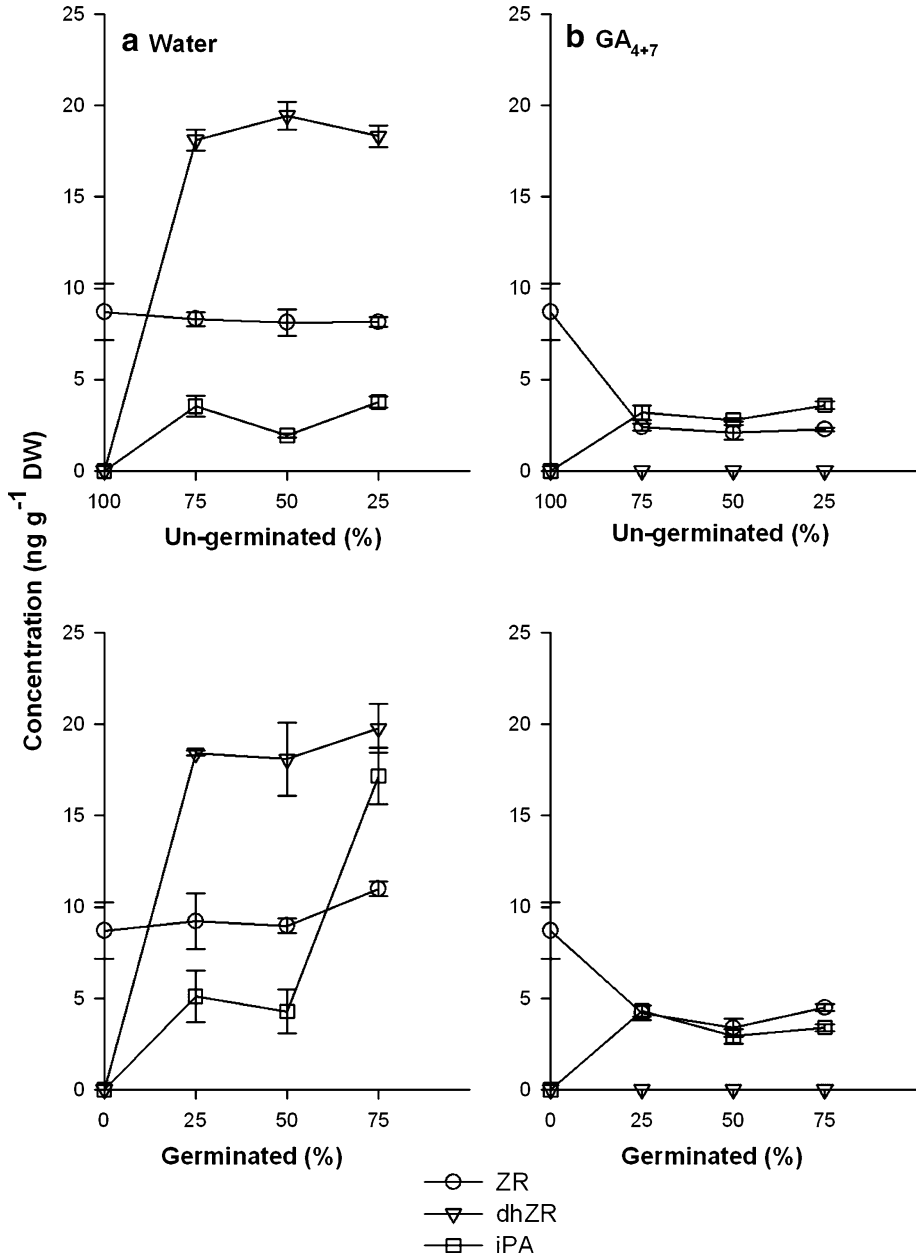

Fig. 9 Changes in cytokinins in both germinated and ungerminated seeds imbibed at 8°C in either water or GA_{4+7} in the absence of light. Seeds were collected at 0, 25, 50, and 75% germination and also their ungerminated counterparts at the same intervals. **a** Water. **b** GA_{4+7}. Values are means ± SE of three replicates

(2003) and Yamauchi and others (2004) have demonstrated that GA_4 is the major hormone for *Arabidopsis* seed germination, whereas GA_1 plays a minor role. However, in *Arabidopsis etr1-2* mutant seeds (ethylene insensitivity), GA_1, not GA_4, is the major GA associated with seed germination (Chiwocha and others 2005). In addition, GA_1 and GA_3 were also accumulated in shoot tips of *Brassica napus* during a cold period, indicating roles in vernalization (Zanewich and Rood 1995). These studies, in combination with our results and other studies (Hedden and Kamiya 1997), indicate that candidate gibberellins controlling certain plant growth are variable and dependent on species, tissues, developmental stages, endogenous hormone interactions, and environmental conditions.

GA and ABA Interaction

The antagonistic roles of GA and ABA in controlling germination can occur through a direct or an indirect interaction or both. Direct actions include the interaction between their metabolism, whereas indirect roles would be the opposite effect of GA and ABA on genes that regulate seed germination. There is a great deal of evidence to support the indirect action of GA and ABA on seed germination. GA and ABA have opposite effects on genes encoding for endo-β-mannanase, β-1,3-glucanase, α-amylase, and expansin (Leubner-Metzger and others 1996; Chen and Bradford 2000; Wu and others 2001; Leubner-Metzger 2002; Koornneef and others 2002; Wu and

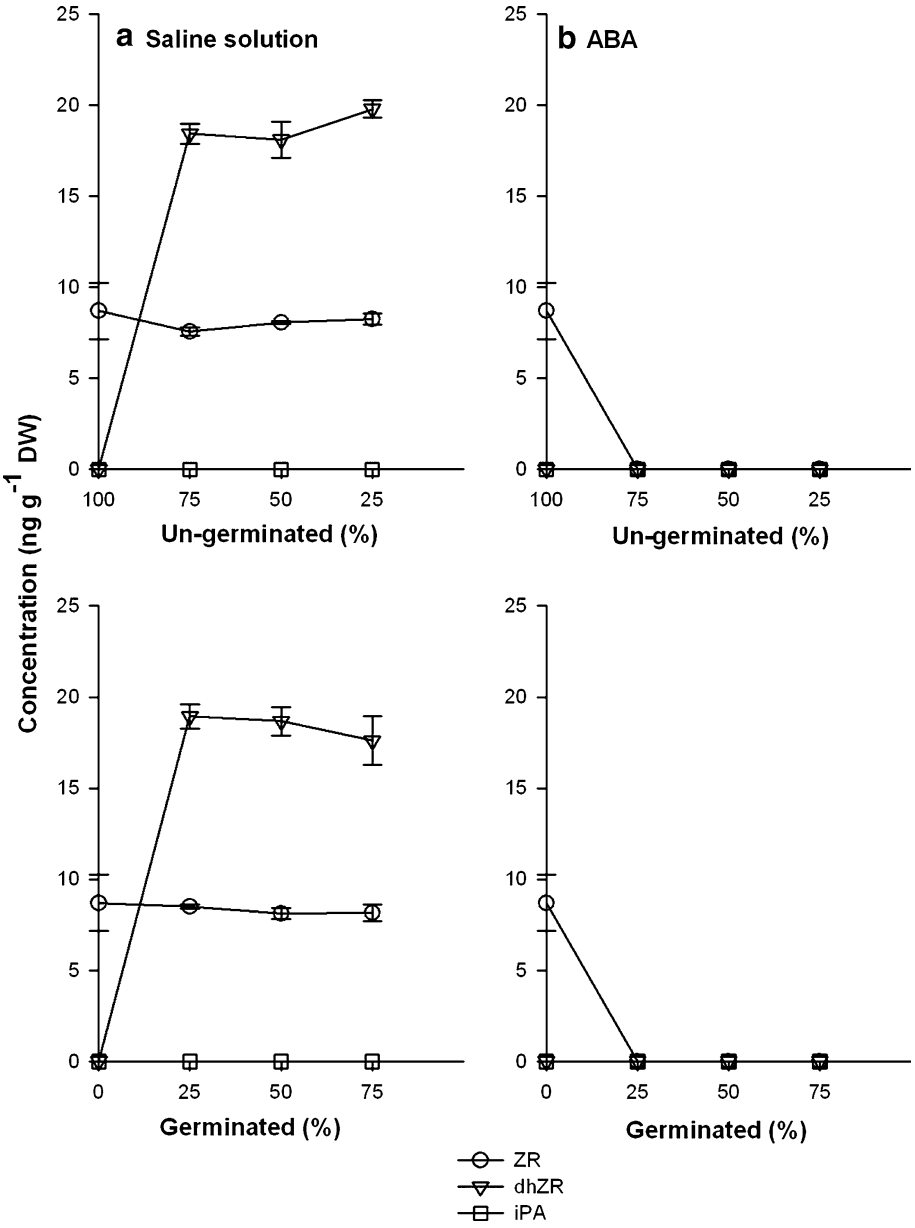

Fig. 10 Changes in cytokinins in both germinated and ungerminated seeds imbibed at 8°C in either the saline solution or ABA in the absence of light. Seeds were collected at 0, 25, 50, and 75% germination and also their ungerminated counterparts at the same intervals. **a** Saline solution. **b** ABA. Values are means ± SE of three replicates

Bradford 2003). In *Arabidopsis*, this indirect action of GA and ABA was also shown in the expression of several ABRE-containing genes (Ogawa and others 2003). However, there is less evidence to support the direct antagonism of ABA and GA.

GA_3 enhances seed germination by reducing ABA levels in lettuce (Toyomasu and others 1994; Gonai and others 2004), whereas in *Arabidopsis*, exogenous GA_4 had no effect on endogenous ABA (Ogawa and others 2003). In our study, GA_{4+7}-imbibed seeds had a higher level of ABA than seeds imbibed in water, which rules out the possibility that GA affects ABA accumulation. Although GA_{4+7} had no effect on ABA accumulation, we did observe that GA_{4+7} affected the ABA catabolic pathway compared to water-imbibed seeds. In water-imbibed seeds, ABA was degraded to DPA, whereas in GA_{4+7}-imbibed seeds, ABA was catabolized to ABA-GE. This finding indicates that GA alters ABA catabolism and changes it from the major 8′ hydroxylation pathway to the ABA-GE conjugation pathway. ABA-GE is assumed to be an irreversible inactive metabolite of ABA (Cutler and Krochko 1999), although some research has suggested that it can be hydrolyzed to ABA (Sauter and others 2002). In *Arabidopsis* and lettuce seeds, the ABA-GE conjugation pathway has been proposed to be the major pathway for ABA degradation (Chiwocha and others 2003, 2005). From our studies we found that this pathway is activated in seeds imbibed in either GA_{4+7} or ABA, whereas the 8′ hydroxylation is the preferred pathway.

It has been proposed that in barley GA accumulation is inhibited by ABA (Jacobsen and others 2002); however, no direct evidence was obtained to support this hypothesis. We observed that GA_4 levels were lower in ungerminated seeds imbibed in ABA than ungerminated seeds imbibed in water (Fig. 6). This observation supports the hypothesis that ABA has an inhibitory effect on GA accumulation. Although a lower level of GA_4 was detected in ungerminated seeds imbibed in ABA compared to water, GA_4 levels in germinated seeds imbibed in ABA and water were nearly identical (Fig. 6). Based on these results we postulate that seeds overcome exogenous ABA by accumulating GA_4. This ABA-dependent GA requirement was also shown in an *Arabidopsis* mutant (Debeaujon and Koornneef 2000). Because ABA inhibits GA_4 accumulation, a longer imbibition time is required to attain the required level of GA to stimulate germination. Therefore, this may be one mechanism whereby ABA inhibits seed germination. In sorghum, the inhibitory effect of ABA on the expression of the GA 20-oxidase gene, the crucial gene in GA biosynthesis, also supports this antagonistic role of ABA on GA accumulation (Pérez-Flores and others 2003).

Auxins and Cytokinins

Auxin plays a major role in controlling cell elongation in isolated stem and coleoptile; however, there is no direct evidence that it is involved in seed germination. Recently, a study in *Arabidopsis* revealed that several auxin biosynthesis genes and genes encoding auxin carrier proteins are regulated by exogenous GA_4 during seed germination (Ogawa and others 2003). In addition, IAA level changes during seed germination were also reported in lettuce (Chiwocha and others 2003), *Arabidopsis*, and its *etr* mutant (Chiwocha and others 2005). However, our study did not show a consistent involvement of auxins in canola seed germination. A similar conclusion was reached for the involvement of cytokinins. We detected ZR, DhZR, and iPA during seed germination, but no association could be reached regarding their roles in germination.

Conclusion

The major hormones in controlling *Brassica napus* seed germination are ABA and GA, whereas auxins and cytokinins had little or no effect. Reduced ABA levels and increased GA_4 content are required for canola seeds to germinate at 8°C; however, the ratio between these two hormones may be more important. Although ABA declined in imbibed seeds in all treatments, the catabolic pathways responsible for this decline are different. ABA inhibited GA_4 accumulation, whereas GA had no effect on ABA accumulation; however, GA alters the ABA catabolic pathway. Both ABA catabolism and GA accumulation are reduced by salinity.

The number of seeds that germinate over time follows a sigmoidal distribution. Thus, although genetically identified, the seeds are vastly physiologically different because all of the seeds on plants do not ripen at the same time and under the same environmental conditions. In the late phase the germination rate is much slower compared with the exponential stage, and the germination slows in the station stage. Factors that control germination increase include germination-promoting hormones (for example, GAs), a decrease in the germination inhibitory hormones (for example, ABA), the breakdown of the seed coat, and enzymes involved in the cell cycle and breakdown of cell reserves, to name a few. Because not all seeds were created equal, all of the above factors result in a sigmoid pattern of germination; therefore, it is not unexpected that there are different germination responses to phytohormones and environmental constraints for different seed samples within the same population.

Acknowledgments This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) strategic grant to LVG.

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