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Comprehensive Hormone Profiling in Developing Arabidopsis Seeds: Examination of the Site of Abscisic Acid Biosynthesis, Abscisic Acid Transport and Hormone Interactions.

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Abbreviations: ABA glucosyl ester, ABA-GE; cytokinin, CK; days after flowering, DAF; dihydrophaseic acid, DPA; gibberellin, GA; jasmonate, JA; jasmonate-isoleucine, JA-Ile; liquid chromatography-electrospray ionization-tandem mass spectrometry, LC-ESI-MS/MS; neo phaseic acid, neoPA; phaseic acid, PA; nine-*cis* epoxycarotenoid dioxygenase, NCED; salicylic acid, SA; 7'-hydroxy ABA, 7'-OH ABA; *trans*-zeatin, tZ.

Abstract

Abscisic acid (ABA) plays important roles in many aspects of seed development, including accumulation of storage compounds, acquisition of desiccation tolerance, induction of seed dormancy, and suppression of precocious germination. Quantification of ABA in the F_1 and F_2 populations originated from crosses between wild type and an ABA-deficient mutant *aba2-2* demonstrated that ABA was synthesized in both maternal and zygotic tissues during seed development. In the absence of zygotic ABA, ABA synthesized in maternal tissues was translocated into the embryos and partially induced seed dormancy. We also analyzed the levels of ABA metabolites, gibberellins, indole-3-acetic acid (IAA), cytokinins, jasmonates and salicylic acid (SA) in the developing seeds of wild type and *aba2-2*. ABA metabolites accumulated differentially in the silique and seed tissues during development. Endogenous levels of SA were elevated in *aba2-2* in the later developmental stages, whereas that of IAA was reduced compared to wild type. These data suggest that ABA metabolism depends on developmental stages and tissues, and that ABA interacts with other hormones to regulate seed developmental processes.

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Introduction

Seed development is the process during which a mature dry seed is formed following fertilization. Basic embryonic pattern formation occurs during the early stage of seed development called morphogenesis (Meinke 1995; Raz et al. 2001). The latter stage, often referred to as maturation, includes embryo growth, seed filling, reserve accumulation, desiccation and quiescence (Finkelstein 2004; Gutierrez et al. 2007). Abscisic acid (ABA) is a plant hormone that plays crucial roles during seed development, especially during maturation (Finkelstein et al. 2002; McCarty 1995). In the middle stage of seed development (early maturation), when embryo growth and seed filling take place, the accumulation of many seed storage compounds is associated with an increase in ABA content (Finkelstein 2004; Finkelstein et al. 2002). In the final stage of seed development (late maturation), the seeds (embryo) acquire desiccation tolerance and become quiescent (McCarty 1995). Loss of seed desiccation tolerance can be observed in a class of Arabidopsis mutants such as *abi3*, *lec1* and *fus3* (To et al. 2006). These mutants accumulate less seed storage proteins, undergo part of the germination program during seed maturation, and germinate prematurely if excised from developing siliques. ABA sensitivity of the seeds in terms of suppression of germination is reduced in *abi3* and to a lesser extent in *lec1,* whereas that of *fus3* is comparable to wild type, suggesting ABA-dependent and -independent mechanisms regulate seed maturation (Meinke et al. 1994; Nambara et al.

2000; Parcy et al. 1997). Although Arabidopsis ABA-deficient mutants are clearly non-dormant, their seeds do not exhibit reduced storage accumulation, desiccation intolerance or premature germination (Koornneef et al. 1989; Meurs et al. 1992; Parcy et al. 1994; Raz et al. 2001). In contrast, ABA deficient mutants in maize are viviparous (McCarty 1995). It appears that the degree to which ABA contributes to the seed maturation processes differs depending on the plant species.

It has been reported that endogenous ABA levels peak in siliques in the middle stage of seed development, with a second small peak of ABA accumulation observed in the late developmental stage (Gazzarrini et al. 2004; Karssen et al. 1983). Reciprocal crossing between wild type and an ABA-deficient mutant indicated that the majority of ABA accumulated in siliques during the middle stage of development originated from maternal tissues (Karssen et al. 1983). The same study showed that, in the later peak, the ratio of ABA synthesized in zygotic tissues increased relative to the middle stage while that in siliques decreased. The F_1 seeds generated by crossing ABA-deficient ovules with wild-type pollen released a thinner mucilage layer on imbibition compared to wild type, but retained wild type seed dormancy, indicating that ABA synthesized in different tissues at different stages has distinct physiological functions (Karssen et al. 1983).

Other hormones in addition to ABA are involved in seed developmental processes, and hormone interactions in relation to seed dormancy and

germination have been discussed (Finkelstein et al. 2008; Finkelstein 2004). In addition to its important role during morphogenesis, indole-3-acetic acid (IAA) affects seed ABA sensitivity during germination (Liu et al. 2007). Gibberellin (GA) is required to induce seed germination and mutants defective in GA biosynthesis germinate poorly (Bewley 1997; Finkelstein et al. 2008). ABA negatively regulates GA biosynthesis in developing and imbibed seeds, and suppression of GA biosynthesis by ABA is required for the induction and/or maintenance of seed dormancy (Seo et al. 2006). Similarly, premature germination of *fus3* is associated with up-regulation of GA biosynthesis in the mutant (Curaba et al. 2004). Mutants insensitive to ethylene, such as *etr1* and *ein2,* are hypersensitive to ABA during germination whereas the constitutive ethylene response mutant *ctr1* is insensitive to ABA (Beaudoin et al. 2000; Ghassemian et al. 2000). Endogenous ABA levels are elevated in *etr1* compared to that in wild type, suggesting that ethylene signals affect ABA levels (Chiwocha et al. 2005).

Physiological responses mediated by hormones are often related to changes in their endogenous concentrations. Thus, precise quantification of the hormone levels is required in order to understand hormone responses. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-EIS-MS/MS) has been applied for comprehensive hormone analysis (Chiwocha et al. 2003; Chiwocha et al. 2005; Kojima et al. 2009; Preston et al. 2009). This method is suitable for the simultaneous quantification of several

hormones that have similar chemical properties. In the present study, we employed LC-ESI-MS/MS to quantify hormones and related compounds during seed development. We examined the contribution of ABA synthesized in maternal and zygotic tissues on ABA accumulation in developing Arabidopsis seeds. Transport of ABA from the maternal to zygotic tissues was further examined. We also investigated the effects of ABA deficiency on the accumulation of other hormones and hormone metabolites and discuss hormone interactions and their physiological roles during seed development.

Results

ABA accumulation in seeds and silique tissues during seed development

Previous studies have shown in Arabidopsis that endogenous ABA levels in siliques reach a maximum in the middle of development, at around 9-10 days after flowering (DAF), and a second peak of ABA accumulation takes place late in development at around 15-16 DAF (Gazzarrini et al. 2004; Karssen et al. 1983; Okamoto et al. 2006). In most cases, however, ABA levels were analyzed without separating seeds from siliques during the course of development. To have a clearer understanding of ABA-mediated physiological responses in seeds, the precise site of ABA accumulation needs to be determined.

We first quantified ABA levels in whole siliques according to developmental stages after flowering (Fig. 1). In our growth condition, seeds

were completely mature at around 21 DAF, green embryos were almost fully expanded at 9 DAF and seed coat browning and chlorophyll breakdown were visible around 15 DAF. ABA levels in whole siliques were constant during the early stage of development (~6 DAF). We observed the first peak of ABA accumulation at 9 DAF, which corresponds to the middle stage of development. ABA levels decreased at 12 DAF, and then increased again toward the end of development. This pattern of ABA accumulation is similar to that previously reported (Gazzarrini et al. 2004; Okamoto et al. 2006). Next, we separated siliques into the seeds and other remaining parts (silique envelopes including the pedicles, receptacles, valves, replums, septa and funiculi), and ABA levels in each part were analyzed (Fig. 1). Separated silique tissues were not analyzed before 9 DAF because of the difficulty in isolating intact seeds from other parts at earlier stages; the seeds are soft and easily damaged as they contain liquid endosperm cells. At 9 DAF, ABA contents per gram dry weight (DW) were approximately twenty times higher in the seeds compared to the levels in silique envelopes. Considering that the ratio in DW of the seeds versus silique envelopes was approximately 1:1.3 at 9 DAF, most of the ABA accumulated in siliques was localized in the seeds at this stage. At 12 DAF, ABA levels in the seeds decreased to one third of the levels at 9 DAF, and the levels were maintained until the end of development. By contrast, ABA levels in silique envelopes increased more than twenty times compared to the levels at 9 DAF toward the end of development (ratios

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in DW of the seeds versus silique envelopes varied 1:1 to 1:0.5 at 21 DAF).

These results indicates that the first peak of ABA levels in 9 DAF siliques can

be attributed to ABA accumulation in the seeds, and the increase in ABA

levels toward the end of development attributed to ABA accumulation in

silique envelopes.

ABA synthesis in maternal and zygotic tissues

The F_1 seeds generated by crossing ABA-deficient ovules with wild-type

pollen, which develop on an ABA-deficient mother plant, consist of an

ABA-deficient seed coat and wild-type (heterozygote) embryo and endosperm.

Previous ABA measurements for siliques containing such F_1 seeds indicated

that ABA was synthesized in both maternal and zygotic tissues during seed

development (Karssen et al. 1983). In mid-seed development, most of the

ABA accumulated in siliques originated from maternal tissues, whereas the

ratio of ABA synthesized in zygotic tissues was relatively high at the end of

seed development. As our results indicated that differential accumulation of

ABA in seeds and silique envelopes during development (Fig. 1), more careful

investigation was required to clarify the site of ABA biosynthesis during seed

development. We examined ABA accumulation in F_1 seeds derived from a

cross using the ABA-deficient mutant *aba2-2* as the mother plant with pollen

from wild type (hereafter referred to as *aba2-2* ♀ × WT ♂) after separating

them from siliques (Fig. 2). ABA levels in the F_1 seeds were comparable to

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that in wild type or slightly reduced relative to wild type at 9 and 15 DAF, respectively. These data suggest that ABA was synthesized mostly in zygotic tissue (i.e. embryo and endosperm) of the F_1 seeds at both stages.

The F_2 seeds derived from the F_1 between wild type and an ABA-deficient mutant, which develop on wild-type (heterozygote) mother plants, contain wild-type seed coat, but zygotic tissues of the F_2 segregate 3:1 for wild type and ABA-deficient type. If ABA is synthesized in zygotic tissues as suggested by the experiment described above, it is expected that ABA levels in the F_2 population segregate 3:1 for normal (wild type) and reduced (ABA-deficient) levels according to their genotypes in zygotic tissues. To determine the effect of ABA deficiency in zygotic tissues on ABA accumulation in seeds, we attempted to measure ABA levels in the individual F_2 seeds (Fig. 3). We detected the peak of endogenous ABA on LC-ESI-MS/MS from one seed and could quantify the levels in the individual F_2 seeds. Similar values for ABA content per seed were obtained when 1, 10, 20 and 50 seeds of wild type at 9 DAF were used for quantification, although the values were sometimes more variable (Fig. 3A). This variability did not appear to depend on the position of the seeds within a silique or the siliques on a plant (data not shown). Note that ABA levels in wild-type seeds at 9 DAF presented in Fig.3 (around 5 pg/seed) were lower than those presented in Fig.2 (around 14 pg/seed). This is probably because the seeds used for these two experiments were derived from plants that were not grown together and

reflects differences in growth and sampling conditions. Furthermore, ABA levels in seeds change drastically around 9 DAF as shown in Fig.1, and this might contribute the variations. As in the case of wild type, ABA levels in *aba2-2* determined from 1, 10 and 20 seeds were comparable (Fig. 3A). Importantly, reduced ABA levels in *aba2-2* compared to wild type were apparent even when one seed was used for the quantification. From these results, it appeared possible to distinguish wild type and *aba2-2* in the F_2 population if their ABA levels segregate according to their genotypes. ABA levels measured for individual wild-type and F_2 seeds are presented in Fig. 3B and 3C, respectively. In total 24 seeds (8 seeds from three independent siliques) for wild type and 80 seeds (40 seeds each from two independent siliques) for the F_2, respectively, were analyzed. In the F_2 population, although one seed clearly contained reduced ABA level compared to wild type, a clear-cut border between ABA levels in wild type and *aba2-2* could not be defined. Fig. 3D shows distribution of ABA levels relative to average in wild-type and the F_2 populations. Similar distribution patterns were observed for individual seeds from wild-type and the F_2 population, indicating that both populations accumulated ABA in a similar way. This indicates that ABA was synthesized mainly in maternal tissues when zygotic tissues, but not maternal tissues, were ABA-deficient.

Similar analyses of ABA levels in wild-type, *aba2-2* and the F_2 seeds were carried out at 15 DAF (Fig. 4). Consistent with the data presented in

Fig.1, ABA levels in wild type seeds were relatively low at 15 DAF compared to those at 9 DAF (Fig. 4A). Variations in the ABA levels determined from one seed were prominent in this stage compared to that in 9 DAF (Fig. 4A and 4B). Comparable values with overlapping standard deviations for ABA content per seed were obtained when 1, 10, 20 and 50 seeds were analyzed with replicates (Fig. 4A). The *aba2-2* seeds contained significantly reduced ABA levels compared to wild type, and the levels were much lower than those at 9 DAF (Fig. 3A). These data demonstrated that ABA levels in wild type and *aba2-2* could be distinguished, if ABA levels in the F_2 seeds segregate according to the genotypes of their embryonic tissue. ABA levels in wild-type and the individual F_2 seeds are shown in Fig. 4B and 4C, respectively. Although variations in ABA levels could be observed in the F_2 population, as in wild type, approximately one-fourth (19 out of 80) of the seeds contained clearly reduced ABA levels compared to the minimum ABA levels in wild type (estimated as approximately 2 pg/seed). The relative distribution of ABA levels differed between the two populations; the F_2 population included seeds with lower ABA contents compared to wild type (Fig. 4D). These data indicate that reduced ABA levels in zygotic tissue segregate from wild type ABA levels in the F_2 populations at 15 DAF. In other words, ABA synthesized in zygotic tissues contributed to the accumulation of ABA in seeds in this developmental stage. Note that the seeds segregating from wild type in terms of ABA levels contained relatively high levels of ABA compared to the seeds

developed on *aba2-2* homozygous plants, indicating that maternal ABA also accumulated in the seeds in this stage.

Localization of ABA within a seed

Our results suggested that ABA is synthesized in maternal and zygotic tissues at both 9 and 15 DAF. Although it is difficult to define the actual site of ABA biosynthesis in wild type, it is feasible to examine possible relocalization of ABA after synthesis in a particular tissue. To determine the site of ABA accumulation within a seed, we separated wild-type seeds into embryos and the fraction containing testa and endosperm. It was impossible to further dissect the fraction into endosperm and testa. Both 9 and 15 DAF seeds accumulated most of their ABA in embryos (Figure 5A). The analysis of F_2 seeds from *aba2-2* ♀ × WT ♂ (Fig.3) indicated that ABA was synthesized mainly in maternal tissues when zygotic tissues, but not maternal tissues, were ABA-deficient. We therefore examined whether preferential accumulation of ABA could be observed in embryos derived from *aba2-2* ♀ × WT ♂ F_2 seeds. Segregation of ABA levels for wild type and *aba2-2* was not observed in either the embryos or testa/endosperm from the F_2 population (Fig. 5B), indicating that ABA synthesized in the maternal tissues was translocated into the embryos. Note that ABA levels in the embryos and testa/endosperm shown in Fig. 5B were relatively high compared to those presented in Fig. 5A, probably because seeds were

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harvested from plants grown independently as mentioned before.

Effects of ABA deficiency in maternal and zygotic tissues on seed dormancy

It has been reported that ABA synthesized in zygotic tissues plays a central role in the induction and maintenance of seed dormancy (Karssen et al. 1983). To examine the contribution of ABA synthesized in maternal and zygotic tissues on seed dormancy in our experimental conditions, we performed germination tests with the *aba2-2* ♀ × WT ♂ F_1 seeds. Freshly harvested F_1 seeds exhibited primary dormancy, as did wild type, while *aba2-2* was clearly non-dormant (Figure 6A). This result confirms that ABA synthesized in zygotic tissues is required for the induction and maintenance of dormancy. In accordance with this, approximately 25% of the F_2 derived from the F_1 between wild type and *aba2-2* germinated without displaying primary dormancy (Figure 6B). It is noteworthy, however, that the peak of germination observed in the F_2 population was delayed by one day compared to that of wild type, suggesting that maternal ABA can influence seed dormancy to some extent.

Profiling hormones and hormone metabolites in developing seeds

To obtain more insight into ABA homeostasis, we determined the profiles of ABA metabolites during seed development. The levels of phaseic acid (PA), dihydrophaseic acid (DPA), neo phaseic acid (neoPA), 7'-hydroxy ABA (7'-OH

ABA) and ABA glucosyl ester (ABA-GE) were analyzed during seed development (Fig. 7). At this time, internal standards were not available for precursors and thus we focused on the ABA catabolism metabolites. The accumulation of PA and DPA, which are the major ABA catabolism pathway products in Arabidopsis (Kushiro et al. 2004; Saito et al. 2004), was different to that of ABA in whole siliques over the course of seed development (Fig. 7B). Nevertheless when measurements were carried out on siliques separated into seeds and envelopes, PA and DPA were more abundant in seeds at 9 DAF and in envelopes at 21 DAF, similarly to ABA (Fig. 7B). The levels of neoPA and 7'-OH ABA in whole siliques peaked at 9 DAF, and these compounds were mostly located in the seeds, like ABA (Fig. 7B). However, no increase in neoPA and 7'-OH ABA levels was observed at the end of development. Although ABA-GE was detected in developing siliques, it could not be quantified due to the presence of an impurity (data not shown). We then compared the levels of ABA metabolites in developing seeds separated from siliques between wild type and *aba2-2* at 9 and 15 DAF (Fig. 7C). The levels of all metabolites were reduced in *aba2-2* relative to wild type at these two time points, but to different degrees depending on the metabolite.

GA regulates seed dormancy and/or germination antagonistically to ABA (Bewley 1997; Finkelstein et al. 2008; Koornneef et al. 1982). Although the physiological roles of GA during seed development are not well understood, it has been reported that GA biosynthesis is up-regulated in ABA-deficient

mutants and other mutants that show premature or precocious germination during seed development (Curaba et al. 2004; Seo et al. 2006; White et al. 2000). These data indicate that suppression of GA biosynthesis is required for the induction of seed dormancy. Most of the non-13-hydroxylated GAs analyzed, GA_{24}, GA_9, GA_4, GA_{51} and GA_{34}, were detected in the early stages of seed development, but were hardly detectable in later stages (Fig. 8A). In contrast, 13-hydroxy GAs such as GA_{53}, GA_{20}, GA_1, GA_{29} and GA_8 were relatively abundant in the mid-development although some of them were also detected in at early stages (Fig. 8A). Among the 13-hydroxy GAs analyzed, GA_{19} showed a different pattern of accumulation during development, and the levels were high compared to other GAs (Fig. 8A). The distribution of 13-hydroxy GAs in seeds and silique envelopes was compared at 9, 15 and 21 DAF (Fig. 8B); non-13-hydroxy GAs were not analyzed because their levels were very low in whole siliques at these stages. At 9 DAF, GA_{20}, GA_1 and GA_{29} were most abundant in seeds. GA_{19} was detected mainly in silique envelopes at these stages. GA_8 was detected in both seeds and silique envelopes at 9 DAF, but only in silique envelopes at later stages. Our previous study demonstrated the up-regulation of GA-biosynthesis and GA-inducible genes in *aba2-2* during seed development (Seo et al., 2006). GA levels in developing seeds at 9 and 15 DAF were compared between wild type and *aba2-2*. However, no significant difference in their levels was observed (data not shown).

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Endogenous levels of indole-3-acetic acid (IAA), cytokinins (CKs), Jasmonates (JAs) and salicylic acid (SA) in developing seeds were analyzed to discuss their physiological roles especially in relation to interaction with ABA (Fig. 9). IAA accumulated to high levels in siliques from early to mid-seed development with a strong peak at 6 DAF (Fig. 9A). The peak of IAA accumulation appeared to take place in seeds because IAA accumulated mostly in seeds at 9 DAF. In *aba2-2*, IAA levels in seeds were reduced to approximately half of the wild type levels at 15 DAF (Fig. 9B). Among the CKs analyzed in this experiment, *trans*-zeatin (tZ) was predominant during seed development, although the levels were relatively low throughout the development compared to other hormones (Fig. 9C). Similar to IAA, tZ levels peaked at 6 DAF, and appeared to be distributed mainly in seeds (Fig. 9C and D). No clear difference in CK levels was observed in *aba2-2* compared to wild type (data not shown). JA and jasmonate-isoleucine conjugate (JA-Ile) showed similar patterns of accumulation in whole siliques during the course of development; their levels were relatively high in flower buds and flowers, then decreased in mid-development, before increasing in later stages (Fig. 9A). Interestingly, however, JA and JA-Ile accumulated differentially in the seeds and silique envelopes. JA accumulation in later stages took place in silique envelopes, whereas JA-Ile accumulated in both seeds and silique envelopes in these stages. JA and JA-Ile levels did not significantly differ between wild type and *aba2-2* (data not shown). The pattern of SA

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accumulation in whole siliques resembled that of JA and JA-Ile being relatively high at early stages, decreasing in mid-development and subsequently increasing in late stages (Fig. 9A). SA accumulated mainly in silique envelopes towards the end of silique development (Fig. 9A). At 15 DAF, SA levels in seeds were approximately two times higher in *aba2-2* compared to wild type (Fig. 9B).

Discussion

Maternal and zygotic synthesis of ABA

As reported previously, we observed a peak of ABA accumulation in whole siliques in the middle stage of seed development (9 DAF in our condition) (Fig. 1). ABA levels in siliques decreased at 12 DAF and then increased again towards the end of development (Fig. 1). When siliques were separated into the seeds and envelopes, ABA was detected mostly in the seeds in the middle stage (9 DAF) and in the envelopes in the late stage (18 and 21 DAF) (Fig. 1). It has been demonstrated that nine-*cis* epoxycarotenoid dioxygenase (NCED), encoded by five *AtNCED* genes in Arabidopsis, is the key regulatory enzyme in ABA biosynthetic pathway (Nambara and Marion-Poll 2005). Previous reports showed that in whole siliques *AtNCED6* and *AtNCED9* were highly expressed at mid-seed development, whereas the expression of *AtNCED2* and *AtNCED3* was high at later stages (Okamoto et al. 2006). This suggests that *AtNCED6* and *AtNCED9* contribute to the accumulation of ABA in seeds

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in mid-development, and *AtNCED2* and *AtNCED3* contribute to increased ABA levels in silique envelopes at later stages. In agreement, the expression of *AtNCED6* and *AtNCED9* in seeds was detected mostly in mid-seed development (Lefebvre et al. 2006). In addition, the mutants defective in *AtNCED6* and *AtNCED9* contained lower ABA levels in dry seeds compared to wild type, although the levels in developing seeds were not determined (Lefebvre et al. 2006). In contrast, *AtNCED2* and *AtNCED3* are the main *AtNCED*s expressed in silique envelopes in late development (Supplementary Fig. S1). However, phenotypes of mutants defective in *AtNCED2* and *AtNCED3* have not yet been characterized for potential physiological roles of ABA in this tissue.

By analyzing ABA levels in siliques containing the F_1 seeds generated by crossing an ABA-deficient female and wild-type male, Karssen et al. (1983) demonstrated that ABA accumulated in siliques in mid-silique development was synthesized in maternal tissues and at later stages in zygotic tissues. On the contrary, our ABA measurements in the F_1 (*aba2-2* ♀ × WT ♂) seeds isolated from siliques clearly showed that ABA was synthesized mainly in zygotic tissues at both mid (9 DAF) and late (15 DAF) developmental stages (Fig. 2). Nevertheless, ABA measurements for individual F_2 seeds derived from the same cross indicated that ABA was synthesized mainly in maternal tissues in the mid-development (Figs. 3). ABA measurements in the F_2 populations also indicated that significant amount of ABA was synthesized in

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zygotic tissues in the late development even though maternal ABA also accumulated in seeds in this stage (Fig. 4). Although we cannot fully explain the reasons for the contradictory results, our present data indicates that either maternal or zygotic ABA is sufficient to maintain ABA levels in seeds in mid-development. Furthermore our results suggest the presence of a mechanism for compensating ABA levels in response to ABA deficiency in maternal and zygotic tissues. It might be also possible that xanthoxin produced in maternal tissues is required for ABA biosynthesis in embryos: previous study (Karssen et al. 1983) used *aba1* mutant impaired in upstream of xanthoxin production whereas in the present study we used *aba2* mutant defective in the reaction downstream of xanthoxin.

Little is known about the physiological roles of ABA synthesized in different tissues. ABA synthesized in zygotic tissues has a predominant role in the induction and/or maintenance of seed dormancy (Karssen et al. 1983, Fig. 6). Even though maternal ABA accumulated in embryos in mid-seed development when zygotic tissues were ABA-deficient (Figs. 3 and 5), the ABA synthesized in maternal tissues was not sufficient to fully induce seed dormancy (Fig. 6B, also see later in discussion). Specific site and/or timing of ABA biosynthesis might be required for the full induction of seed dormancy. Zygotic tissues are composed of at least two different tissue types, the endosperm and embryo. *AtNCED6* is expressed in the endosperm whereas *AtNCED9* is expressed in the embryo and endosperm, suggesting that ABA is

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synthesized in both tissues (Lefebvre et al. 2006). The precise physiological roles of ABA synthesized in the endosperm and embryo at molecular levels, remain to be evaluated.

Grafting experiments using ABA-deficient mutants in *Nicotiana plumbaginifolia* indicated that part of the ABA synthesized in mother plants is transported to seeds (Frey et al. 2004). This study showed that although ABA transported from mother plants had no effect on dormancy induction it was important for seed developmental processes such as embryo growth, seed pigmentation, seed productivity and so on. In Arabidopsis, maternal ABA affected the thickness of the mucilage layer released from mature seeds on imbibition (Karssen et al. 1983). Furthermore, maternal ABA inhibited precocious germination of *fus3* (Raz et al. 2001). These data indicate that maternal ABA plays roles in several developmental processes. Our present data suggests that ABA synthesized in maternal tissues is translocated into the embryos if zygotic tissues, but not maternal tissues, are ABA-deficient (Fig. 5). In this situation, ABA synthesized in maternal tissues partially induced primary dormancy in the *aba2-2* background (Fig. 6B). It is possible that ABA transported from maternal tissues into embryos also plays a role in dormancy induction in wild type. It will be interesting to understand how such ABA transport is regulated.

Comprehensive hormone profiling in developing seeds

Several pathways for ABA catabolism have been reported (Nambara and Marion-Poll 2005). We observed that ABA, PA, DPA, neoPA and 7'-OH ABA were accumulated differentially in siliques, seeds and silique envelopes over the course of seed development, indicating that these pathways are regulated independently (Fig. 7). Differential accumulation of ABA metabolites has been reported also in *Brassica napus* (Jadhav et al. 2008). Among the proposed ABA catabolism pathways, ABA 8'-hydroxylase encoded by *CYP707A* family has been functionally characterized (Nambara and Marion-Poll 2005). Based on the chemical reaction, it is considered that 7'- and 9'-hydroxylation of ABA are catalyzed by cytochrome P450. It is possible that CYP707As catalyze 7'- and 9'-hydroxylations of ABA in addition to its predominant role for 8'-hydroxylation, but it is also possible that other P450s are specifically involved in 7'- and 9'-hydroxylation. The latter possibility is supported by the observation that the metabolites in each pathway exhibited different spatial and temporal accumulation (Fig. 7).

GA_1 and GA_4 are the major bioactive GAs in Arabidopsis and other plant species. GA_1 is synthesized via the 13-hydroxy pathway whereas GA_4 is synthesized via non-13-hydroxy pathway. In Arabidopsis, the non-13-hydroxy pathway is predominant although physiological roles of the two pathways are not well understood (Yamaguchi 2008). Consistent with a previous report (Hu et al. 2008), non-13-hydroxy GAs were predominant in flower buds, flowers and early developing siliques (Fig. 8). In contrast, the 13-hydroxy pathway

appeared to be more active in mid-seed development relative to the non-13-hydroxy pathway (Fig. 8). Similarly, relatively higher GA_1 levels have been reported in developing Arabidopsis seeds (Curaba et al. 2004). It has been shown that precocious or premature germination of maize and Arabidopsis is dependent on endogenous GA (Raz et al. 2001; White et al. 2000). It can be supposed that GA promotes seed developmental processes, but suppression of GA biosynthesis would be required for the induction of seed dormancy. We have previously reported that the expression of GA-biosynthesis genes, *GA20ox* and *GA3ox*, and GA-inducible genes are up-regulated in the developing seeds of *aba2-2* at 15 DAF, indicating that ABA suppresses GA biosynthesis in late stages of seed development. However, as GA levels were very low no clear increase in GA levels could be detected in *aba2-2* relative to wild type (data not shown).

It has been well documented that IAA plays a crucial role in embryonic pattern formation (Moller and Weijers 2009). IAA levels reached a maximum at 6 DAF, suggesting its role on seed maturation (e.g. embryo growth) (Fig. 9A). Activation of IAA-biosynthesis and -inducible genes by LEC2 has been reported (Stone et al. 2008), however, the physiological role of IAA during seed maturation is not clear. Crosstalk between IAA and ABA during seed germination has been reported (Liu et al. 2007). IAA enhances ABA-mediated inhibition of seed germination, indicating that IAA is a positive regulator of ABA responses. In the present study, we found that IAA levels in *aba2-2* were

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reduced to approximately half those of wild type during late seed development (Fig. 9B). It is possible that positive regulation of IAA levels by ABA, which in turn enhances ABA responses, is a part of the mechanism by which ABA inhibits seed germination.

In developing seeds, tZ was the most abundant if the CKs analyzed in this study (Fig. 9C). It has been reported that mutants defective in CK receptors produce larger seeds compared to wild type (Riefler et al. 2006). Accumulation of tZ at 6 DAF (Fig. 9C), when embryos undergo expansion, might be related to this process. The CK receptor mutants exhibited germination phenotypes reminiscent of ABA-deficient mutants; rapid germination, reduced requirement for light, and reduced sensitivity to far-red light irradiation (Riefler et al. 2006). This might suggest that CKs regulates homeostasis and/or signaling of ABA. We did not observe altered CK levels in *aba2-2* (data not shown), suggesting that ABA did not affect CK metabolism.

Accumulation of JA and JA-Ile in flower buds and flowers (Fig. 9A) is probably related to its function in anther development as reported previously (Ishiguro et al. 2001). It is known that JAs induces senescence and fruit ripening (Creelman and Mullet 1997), suggesting that the accumulation of JA and JA-Ile in fruit (silique envelopes) at late developmental stages might be related to these functions. Studies indicate that JA is converted to JA-Ile to become bioactive (Staswick and Tiryaki 2004). JA accumulated at

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high levels in silique envelopes whereas JA-Ile levels rose in both seeds and silique envelopes during late silique development (Fig. 9A), suggesting that the production of JA-Ile is regulated in a tissue specific manner. JA-insensitive mutants, *jin4* and *jar1*, exhibit an increased sensitivity to ABA during germination (Berger et al. 1996; Staswick et al. 1992), suggesting a possible interaction between JA(s) and ABA. Nevertheless, JA and JA-Ile levels in *aba2-2* were comparable to those in wild type (data not shown), suggesting that ABA does not affect the metabolism of JAs.

Little is known about the physiological roles of SA during seed development. We found that SA levels were high in the developing seeds of *aba2-2* at 15 DAF compared to those of wild type (Fig. 9B). This suggests that ABA negatively regulate SA accumulation during seed development. Interactions between SA and ABA, as well as JA and ABA, during biotic stress responses have been reported (de Torres-Zabala et al. 2007; Yasuda et al. 2008). It is possible that interactions between SA, JA and ABA in developing seeds are related to defense responses to pathogens. Phenotypic characterization of seeds defective in SA, JA and ABA metabolism and responses will be required to determine if this is the case.

Materials and Methods

Plant Materials

Arabidopsis thaliana accession Columbia (Col) and an ABA-deficient mutant

aba2-2 isolated in Col background (Nambara et al. 1998) were used for experiments. Seeds were sown on pots containing vermiculite, watered with nutrient solutions and grown under constant light at 22°C. To collect silique and seed samples at different developmental stages, flowers were tagged at the time of flowering. Siliques that developed in the middle of main stems were used for the experiments. The whole silique samples contained a part of the pedicle. When required, siliques were dissected under a microscope into the seeds and the other silique parts (silique envelopes), which contained pedicles, receptacles, valves, replums, septa and funiculi. Samples were frozen in liquid nitrogen and stored at -80°C until used.

Germination assays

Freshly harvested mature dry seeds were surface-sterilized with a solution containing 5% (v/v) NaClO and 0.05% (v/v) Tween 20, rinsed with water, and sown on 1.2% (w/v) agar plates. Plates were incubated at 22 °C under constant light and germination rates was scored every day. Germination was defined as the protrusion of the radicle from the seed coat.

Small-scale extraction and purification of ABA

For ABA measurements from small number (1-50) of seeds (Figs. 2, 3, 4 and 5), samples placed in PCR tubes were homogenized with beads with Tissue Lyser (QIAGEN) in 100 μl of 80% (v/v) methanol containing 1% (v/v) acetic

acid with d_6-ABA as an internal standard. After extraction at -30°C overnight, methanol containing water was completely evaporated in a SpeedVac (Thermo Fisher) and extracts were dissolved in 200 µl of water contining 1% (v/v) acetic acid. Extracts were applied to Oasis HLB µ Elution plates (Waters) that had been washed with 200 µl of methanol and then by 200 µl of water containing 1% (v/v) acetic acid. The plates were then washed with 200 µl of water containing 1% (v/v) acetic acid and fractions containing ABA were obtained by elution with 200 µl of 70% (v/v) ethanol containing 1% (v/v) acetic acid.

Extraction and purification of multiple hormones and hormone related compounds

Frozen samples were lyophilized before extraction and weighed to calculate hormone levels per DW. Five to ten milligram (DW) samples were used for the analysis of flower buds, flowers, whole siliques, seeds and silique envelopes over the course of seed development (Figs. 1, 7, 8 and 9). For the comparison of hormone levels between wild type and *aba2-2* (Figs. 7 and 9), relatively large amount of samples (around twenty milligrams DW) were analyzed so as to accurately detect differences between the two samples. Extraction and purification of hormones and hormone related compounds were conducted according to (Yoshimoto et al. 2009) with some modifications. Detailed procedures are described in Supplementary Method and

Supplementary Fig. S2.

LC-ESI-MS/MS analysis

Fractions containing hormones were dried up after purifications and dissolved in 30 μl of water containing 1 % (v/v) acetic acid to inject into LC-ESI-MS/MS. Quantification of 14 GAs by LC-ESI-MS/MS was performed as described in (Varbanova et al. 2007). ABA, IAA, iP, tZ, DHZ, JA, JA-Ile and SA were quantified according to (Yoshimoto et al. 2009). ABA-GE was analyzed by ACQUITY UPLC system (Waters)/ Q-Tof premier (Micromass, Manchester, UK) (Saika et al. 2007) or Agilent 1200-6410 system. Conditions and parameters for Agilent 1200-6410 equipped with ZORBAX Eclipse XDB-C18 column (1.8 μm, 2.1 × 50 mm) for analysis of ABA and ABA metabolites are summarized in Supplementary Tables SI and SII.

Internal standards

Internal standards were obtained as described previously (Abrams et al. 2003; Chiwocha et al. 2003; Varbanova et al. 2007; Yoshimoto et al. 2009; Zaharia et al. 2005).

Funding

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Figure legends

Fig. 1 ABA levels during wild-type seed development. ABA levels (ng/g DW) in wild-type flower buds (bud), flowers (0 DAF), whole siliques (3, 6, 9, 12, 15, 18 and 21 DAF), seeds (9, 12, 15, 18 and 21 DAF) and silique envelopes (9, 12, 15, 18 and 21 DAF) were analyzed. Values are means ±SD of three biological replicates. Developmental events corresponding to DAF are indicated below the graph.

Fig. 2 ABA levels in the wild-type (Col), *aba2-2* and F_1 (*aba2-2* ♀ × Col ♂) seeds. Developing seeds from each genotype were isolated from siliques at 9 and 15 DAF, and analyzed for ABA levels (pg/seed). Values are means ±SD of four biological replicates with forty to fifty seeds.

Fig. 3 ABA levels in the wild type (Col), *aba2-2* and F_2 (*aba2-2* × Col) seeds at 9 DAF. (A) ABA levels (pg/seed) were analyzed from 1, 10, 20 and 50 seeds of wild type, and from 1, 10 and 20 seeds of *aba2-2*. Values are means ±SD of the number of biological replicates (rep) indicated below the graph. For ABA measurements from one seed, seeds produced in independent siliques (a, b and c) were analyzed. For other measurements, seeds produced in several siliques were mixed. (B) ABA levels (pg/seed) in the individual wild-type seeds that were produced in independent siliques (a, b and c). (C) ABA levels

(pg/seed) in the individual F$_2$ (*aba2-2* × WT) seeds. A total of eighty seeds, forty seeds from two independent siliques, were analyzed. (D) Distribution of relative ABA levels in the wild type and F$_2$ populations. For wild type, the results for the twenty-four seeds shown in Figs. 3A and 4B were used to generate the graph. For the F$_2$, the results for the twenty-four seeds presented in Fig. 3C were randomly selected and used to generate the graph.

Fig. 4 ABA levels in the wild type (Col), *aba2-2* and F$_2$ (*aba2-2* × Col) seeds at 15 DAF. (A) ABA levels (pg/seed) were analyzed from 1, 10, 20 and 50 seeds of wild type, and from 1, 10 and 20 seeds of *aba2-2*. Values are means ±SD of the number of biological replicates (rep) indicated below the graph. For ABA measurements from one seed, seeds produced in independent siliques (d, e and f) were analyzed. For other measurements, seeds produced in several siliques were mixed. The asterisk indicates that endogenous ABA was not detected in two measurements out of the eight replicates, and the mean value ±SD was calculated for six replicates. (B) ABA levels (pg/seed) in individual wild type seeds that were isolated from 3 independent siliques (d, e and f). (C) ABA levels (pg/seed) in individual F$_2$ (*aba2-2* × WT) seeds. A total of eighty seeds, forty seeds from two independent siliques, were analyzed. The red bars indicate values below wild type minimum ABA levels estimated from Fig. 4B (approximately 2 pg/seed). (D) Distribution of relative ABA levels in the wild type and F$_2$ populations. For wild type, the results for twenty-four seeds

shown in Figs. 4A and 4B were used to generate the graph. For the F_2, the results for twenty-four seeds presented in Fig. 4C were randomly selected and used to generate the graph.

Fig. 5 ABA levels in embryos or testa and endosperm. (A) Developing wild type seeds at 9 or 15 DAF were separated into embryos or testa and endosperm (testa/endosperm), and ABA levels (pg/seed) were analyzed. Values are means ±SD of four biological replicates with twenty seeds for 9 DAF, and four biological replicates with fifty seeds for 15 DAF seeds. (B) ABA levels (pg/seed) in embryos and in testa/endosperm of twenty-four F_2 (*aba2-2* × WT) seeds. Means ±SD (n=24) of ABA levels in the embryos and testa/endosperm of wild type seeds (WT) are also shown.

Fig. 6 Seed dormancy of wild type and mutants with impaired ABA biosynthesis in different seed tissues. (A) Germination of freshly harvested seeds of wild type, *aba2-2* and the F_1 (*aba2-2* ♀ × WT ♂). (B) Germination of freshly harvested wild type, *aba2-2* and the F_2 (*aba2-2* × WT). Values are means ±SD of three biological replicates. Germination was defined as radicle protrusion from the seed coat.

Fig. 7 Profiling of ABA metabolites in developing seeds. (A) Schematic diagram of ABA catabolism pathways. (B) The levels (ng/g DW) of ABA,

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7'-OH ABA, PA, DPA and neoPA in wild type flower buds (bud), flowers (0 DAF), whole siliques (3, 6, 9, 12, 15, 18 and 21 DAF), seeds (9, 15 and 21 DAF) and silique envelopes (9 15 and 21 DAF). Values are means ±SD of three biological replicates. (C) The levels (ng/g DW) of ABA, 7'-OH ABA, PA, DPA and neoPA in wild type and *aba2-2* developing seeds at 9 and 15 DAF. The means ±SD of three biological replicates are presented. The mean values are indicated on the graphs. n.d., not detected in the three replicates.

Fig. 8 GA levels during seed development. (A) GA levels (ng/g DW) in wild type flower buds (bud), flowers (0 DAF) and whole siliques (3, 6, 9, 12, 15, 18 and 21 DAF). Values are means ±SD of three biological replicates. (B) GA levels (ng/g DW) in the seeds and silique envelopes of wild type at 9, 15 and 21 DAF. For (A) and (B), asterisks indicate that the endogenous compounds were detected twice in the three replicates, and means ±SD of the two measurements are presented. Bars without ±SD indicate that the endogenous compounds were detected once in the three replicates. Empty columns indicate that the endogenous compounds were not detected in three replicates.

Fig. 9 The levels of IAA, JAs, SA and CKs during seed development. (A) IAA, JAs and SA levels (ng/g DW) in wild type flower buds (bud), flowers (0 DAF), whole siliques (3, 6, 9, 12, 15, 18 and 21 DAF), seeds (9, 12, 15, 18 and 21

DAF) and silique envelopes (9, 12, 15, 18 and 21 DAF). For JA-Ile in whole siliques at 3, 6, 15, and 21 DAF, and SA in silique envelopes at 21 DAF, means ±SD of two biological replicates are presented. For others, means ±SD of three biological replicates are presented. (B) IAA and SA levels (ng/g DW) in developing seeds of wild type (Col) and *aba2-2* at 9 and 15 DAF. Values are means ±SD of three biological replicates. (C) CK levels (ng/g DW) in flower buds (bud), flowers (0 DAF) and whole siliques (3, 6, 9, 12, 15, 18 and 21 DAF). Endogenous compounds were detected twice in the three biological replicates for isopentenyl adenine (iP) at 0 DAF and tZ at 0 DAF, and the means ±SD of two biological replicates are presented. Endogenous compounds were detected only once in the three biological replicates for dihydrozeatin (DHZ) at 0, 6, 15 and 21 DAF. For others, the means ±SD of three biological replicates are presented. (D) iP and tZ levels (ng/g DW) in wild type seeds and silique envelopes at 9, 12, 15, 18 and 21 DAF. Values are means ±SD of three biological replicates. The asterisk indicates that the endogenous compound was detected twice in the three biological replicates and the mean ±SD of two biological replicates is presented. The bars without ±SD indicate that endogenous compounds were detected only once in three biological replicates. Empty columns indicate that endogenous compounds were not detected in the three biological replicates.