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Hormonal regulation of oil accumulation in *Brassica* seeds: Metabolism and biological activity of ABA, 7′-, 8′- and 9′-hydroxy ABA in microspore derived embryos of *B. napus*

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ABSTRACT

Developing seeds of *Brassica napus* contain significant levels of ABA and products of oxidation at the 7′- and 9′-methyl groups of ABA, 7′- and 9′-hydroxy ABA, as well stable products of oxidation of the 8′-methyl group, phaseic acid and dihydrophaseic acid. To probe the biological roles of the initially formed hydroxylated compounds, we have compared the effects of supplied ABA and the hydroxylated metabolites in regulating oil synthesis in microspore-derived embryos of *B. napus,* cv Hero that accumulate long chain fatty acids. Uptake into the embryos and metabolism of each of the hormone metabolites was studied by using deuterium labeled analogs. Supplied ABA, which was rapidly metabolized, induced expression of oleosin and fatty acid elongase genes and increased the accumulation of triacylglycerols and very long chain fatty acids. The metabolites 7′- and 9′-hydroxy ABA had similar effects, with the 9′-hydroxy ABA having even greater activity than ABA. The principal catabolite of ABA, 8′-hydroxy ABA, also had hormonal activity and led to increased oil synthesis but induced the genes weakly. These results indicate that all compounds tested could be involved in lipid synthesis in *B. napus*, and may have hormonal roles in other ABA-regulated processes.

1. Introduction

The plant hormone abscisic acid (ABA) **1** is involved in the regulation of numerous physiological processes during seed development, including embryo maturation, storage product deposition, inhibition of precocious germination, desiccation tolerance and dormancy (Marion-Poll and Leung, 2006). Levels of ABA **1** in seed tissues at the appropriate developmental time, regulated by a balance of biosynthesis, catabolism and transport, are thought to be critical for proper seed development (Frey et al., 2004). Many of the genes in the ABA biosynthesis pathway and, recently, genes involved in ABA catabolism, have been identified. Arabidopsis transgenics with modified levels of ABA **1** either impaired in ABA biosynthesis or catabolism, exhibit altered profiles of storage product accumulation, dormancy, and/or desiccation tolerance (De Bruijn et al., 1997; Raz et al., 2001; Kushiro et al., 2004; Nambara and Marion-Poll, 2005; Lefebvre et al., 2006).

Research to date has focused on ABA **1** itself as the bioactive compound in the pathway. Less is known about the roles of hydroxylated ABA catabolites in biological processes in which ABA **1** is implicated. Based on extensive genetic and biochemical evidence it has been established that the principal oxidative pathway in ABA catabolism is mediated by the CYP707A class of P450 monooxygenases (Fig. 1) (Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006). These enzymes oxidize ABA **1** to 8′-hydroxy ABA **2** (8′-OH ABA), a labile compound. The isolable 8′-OH ABA catabolite, phaseic acid **3** (PA), is a cyclized rearrangement product, which is found to be inactive in most assays with the exception of inhibition of GA-stimulated alpha amylase in barley (Arai et al., 1999; Zaharia et al., 2005). Interestingly, PA **3** has also been found to be produced by reduction of the corresponding aldehyde, through an oxidation-reduction cycle, and further oxidized compounds have been isolated from plants (Zaharia et al., 2004). Phaseic acid **3** is reduced to dihydrophaseic acid **4** (DPA) which is also biologically inactive.

Microspore-derived (MD) embryos in culture have been shown to mimic developing zygotic embryos at a comparable stage, particularly with respect to the accumulation of storage compounds, proteins and lipids (Holbrook et al., 1990; Taylor and Weber, 1994; Weselake and Taylor, 1999). The facility with which chemical compounds (e.g. hormones, herbicides etc.) as well as various physiological stressors (e.g. osmotic or heat stress) can be applied to MD embryos in liquid culture has resulted in their use as a model system for studying the effects of these manipulations on embryo development for almost twenty years. In studies comparing

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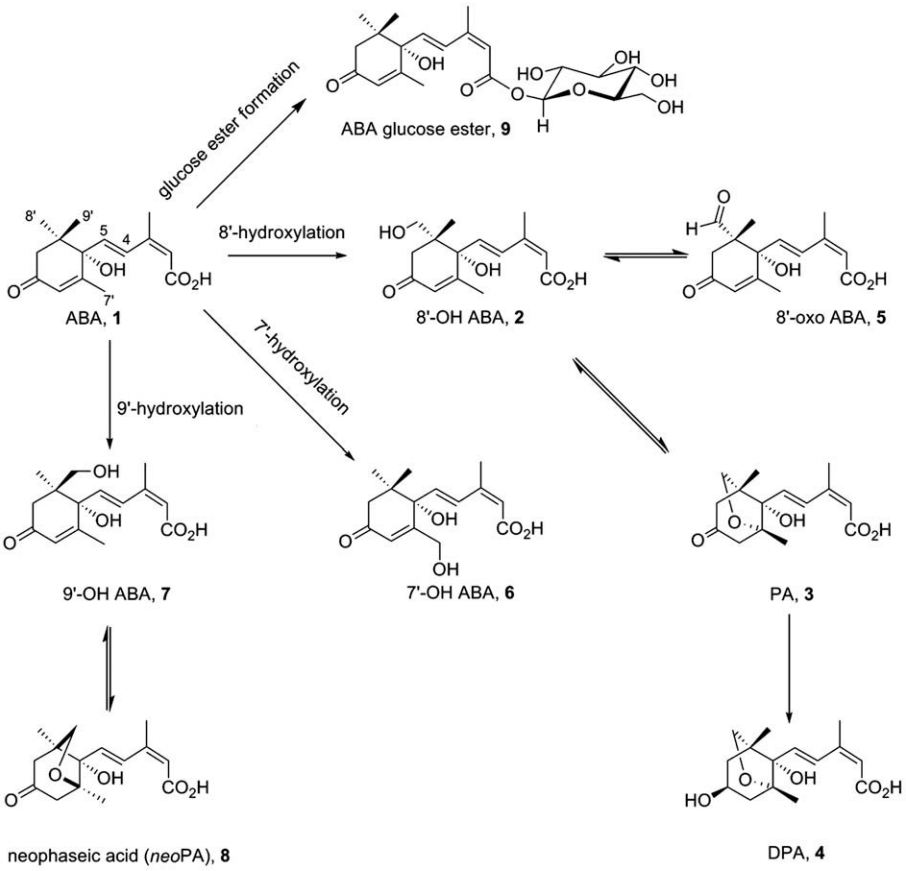

Fig. 1. Metabolism of ABA **1**.

the activity of ABA **1** and the ABA catabolites 8′-OH ABA **2** and PA **3** (Zou et al., 1995; Qi et al., 1998), exogenously supplied (+)-ABA **1** was rapidly taken up by microspore-derived (MD) embryos of high erucic acid cultivars of *Brassica napus*, and resulted in the induction of genes involved in fatty acid modification and oil body and storage protein deposition. More specifically, there was a strong induction of the fatty acid elongase (*FAE1*) gene encoding the condensing enzyme 3-ketoacyl-CoA synthase (3-KCS), the first in a microsomal four-enzyme complex which produces the seed-specific very long-chain monounsaturated fatty acids (VLCMFAs) erucic (22:1) and eicosenoic (20:1) acids. The activity of 8′-OH ABA **2** was probed in greater depth and shown to promote accumulation of VLCMFAs in a manner similar to ABA **1**. In contrast, PA **3** was found to induce fatty acid synthesis genes very weakly, possibly through equilibration to the active form 8′-OH ABA **2**. In a separate time course experiment, (+)-^{3}H-ABA was supplied to MD embryos and the contents of both the tissue and media were analyzed. Those experiments indicated that both PA **3** and DPA **4** accumulated, with the DPA **4** pool predominating at the termination of the experiment (72 h). DPA **4** supplied to the embryos did not induce gene transcription and was therefore considered inactive.

Two additional oxidation pathways are known, arising from hydroxylation of ABA **1** at the 7′-carbon atom or at the 9′-carbon atom, affording 7′-hydroxy ABA **6** (7′-OH ABA), or 9′-hydroxy ABA **7** (9′-OH ABA). These oxidized catabolites have received less attention because the pathways are thought to be minor, and the compounds and analytical standards have not readily been available (Hill et al., 1995; Shimomura et al., 2007). Levels of 7′-OH ABA **6** are significant in a few plant tissues like embryos of western white pine (Feurtado et al., 2004) and ripe blueberries (Abrams et al., unpublished). In barley seed germination, 7′-OH ABA **6** exhibits weak ABA-like activity, and its role in seed development is unknown (Hill et al., 1995). Like 8′-OH ABA **2**, the 9′-hydroxylated compound **7** is in equilibrium with its closed form *neo*PA **8**, a catabolite first found in developing *Brassica* seeds (Zhou et al., 2004). In the report of the identification and biological activity of hydroxylated products at the 9′-carbon atom, the activity of 9′-OH ABA **7** and *neo*PA **8** had also been compared to ABA **1**. The open form of the 9′ hydroxylated compound was found to have activity similar to the parent hormone ABA **1** in induction of the *FAE1* gene. The compound was also more active than ABA **1** in inhibition of seed germination. The metabolic fate of exogenously applied 7′- and 9′-hydroxylated ABA compounds **6** and **7** in plant tissues including MD embryo cultures has not yet been elucidated.

ABA **1** and its catabolites may be conjugated with glucose, with ABA glucose ester (ABA GE) **9** being the predominant form (Zaharia et al., 2005). Although ABA GE **9** is considered to be biologically inactive, it has been proposed that it may rapidly be converted into active ABA **1** by hydrolysis via the AtBG1 β-glucosidase in response to environmental stress (Lee et al., 2006). The relevance of ABA GE **9** during seed development is currently unknown.

The objective of the current study was to probe the role of ABA **1** and its hydroxylated catabolites specifically in the regulation of storage lipid deposition in developing oilseeds. We profiled ABA **1** and its known catabolites in zygotic seeds of *B. napus* from early development onward, to determine the relative pool sizes of the catabolites, particularly at the stage in which storage oil is synthesized. Further, with MD embryos of *B. napus*, we compared the effects of supplied ABA **1** and the hydroxylated compounds on expression of genes involved in fatty acid elongation and oil biosynthesis, on accumulation of VLCMFAs in embryo triacylglycerols (TAGs) and expression of the oleosin gene encoding a protein involved in assembly of oil bodies. We also have monitored the metabolism of each of the supplied compounds to determine their

fate, persistence, and effect on synthesis of endogenous hormones. The results indicate that hydroxylated ABA molecules play a role in ABA-regulated processes in plants and induction of genes associated with embryonic lipid processes in particular.

2. Results

2.1. ABA and catabolites in developing seeds of B. napus

The profile of ABA **1** and the catabolites 7′-OH ABA **6**, PA **3**, DPA **4**, *neo*PA **8** and ABA GE **9** in seeds of *B. napus* is shown in Fig. 2. As the equilibria between open and closed forms can change during extraction and analysis, the PA **3** and *neo*PA **8** levels represent the total of hydroxylated product at either the 8′- or 9′-position, respectively. With this time course, two peaks of ABA **1** are observed, similar to the case in Arabidopsis (Karssen et al., 1983). Interestingly, the levels of ABA GE **9** at 14 DAF are as high as the ABA **1** levels. In the current study, the highest amount of *neo*PA **8** (1000 ng/g dry weight) accumulated at 22 DAF, as the levels of ABA **1** and ABA GE **9** declined. The pool sizes of 7′-OH ABA **6** were low (less than 200 ng/g dry weight) and remained constant throughout.

2.2. Persistence and metabolism of ABA and catabolites in microspore-derived embryos of B. napus

In this study the compounds supplied to MD embryos, (+)-ABA **1** and hydroxylated metabolites, were each labeled with two deuterium atoms, so that their uptake, lifetime and catabolism could be analyzed by mass spectrometric analysis (Fig. 3). The isotopically labeled compounds could be distinguished from endogenous hormones, so the effect of supplied compounds on biosynthesis of ABA **1** and catabolites could readily be assessed. The hormone analog molecules were labeled at positions remote from sites of known oxidation processes so that artifacts from primary isotope effects would be avoided. The ABA d_2-**1** and 8′-OH ABA d_2-**2** molecules were labeled at C-4 and C-5 of the side chain, and the 7′-OH ABA d_2-**6** and 9′-OH ABA d_2-**7** were doubly labeled at C-7′ and C-9′, respectively.

The persistence of the administered compounds was monitored by HPLC analysis of the medium (Fig. 4a–d). Over the course of the experiment, samples were analyzed directly at specified time points, to minimize changes in the equilibria between the open forms 8′-OH ABA d_2-**2** and 9′-OH ABA d_2-**7** and the closed forms PA d_2-**3** and *neo*-PA d_2-**8**, so that the concentrations of the different forms could be estimated. The contents of the medium and tissues, including pools of endogenous unlabeled ABA **1** and metabolites produced by the embryos, as well as the deuterated metabolites arising from metabolism of supplied compounds, were analyzed by HPLC–MS/MS (Figs. 5 and 6 and Supplementary material). The procedures required to prepare samples for HPLC–MS/MS result in altering the equilibrium. Using this technique it was not possible to distinguish between the open versus closed forms of the hydroxylated compounds, but it was possible to quantitate the labeled and unlabeled endogenous hormone and metabolites. It was not possible to quantitate the metabolites of supplied compounds by HPLC, due to interference from background and relatively low levels. As in earlier studies (Zou et al., 1995; Qi et al., 1998), supplied (+)-ABA **1** in the medium was found to be rapidly depleted, with 90% metabolized 24 h after addition (Figs. 4a and 5a). Noteworthy is that PA **3** does not appear to any significant level in the medium, but the DPA **4** level increases dramatically in the medium as ABA d_2-**1** decreases.

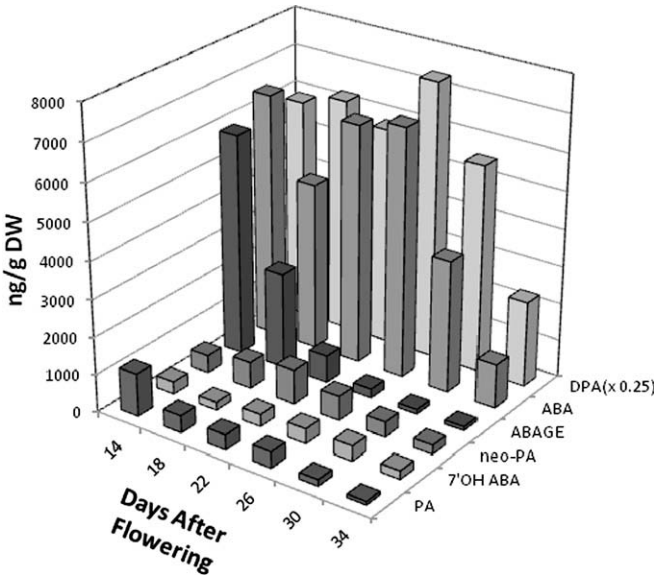

Fig. 2. Levels of ABA and its metabolites in developing seeds of *Brassica napus* cv Quantum. DPA 0.25, dihydrophaseic acid (measured levels divided by 4); ABAGE, ABA glucose ester **9**; 7′-OH ABA, 7′-hydroxy ABA **6**; *neo*PA, *neo*phaseic acid **8**; PA, phaseic acid **3** $n = 3$. (Hormone concentrations with standard deviations are tabulated in Table S1, supplemental material.)

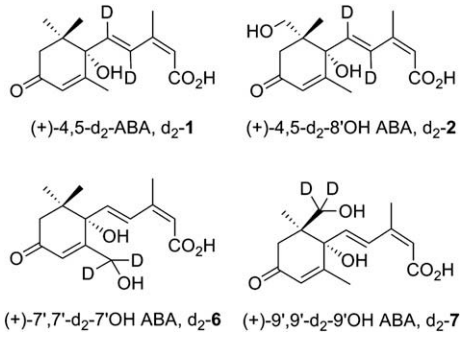

Fig. 3. Chemical structure of the labeled ABA and hydroxylated metabolites supplied to MD embryo cultures.

In the MD embryo extracts the pool of PA d_2-**3** derived from 4,5-d_2-ABA d_2-**1** (Fig. 5b) was five times greater than the ABA d_2-**1** pool by the first sampling time. By 24 h, the pools of PA d_2-**3** and DPA d_2-**4** were equivalent, with the combined catabolites tenfold greater than that of ABA d_2-**1**. By the next time point, the PA d_2-**3** pool was depleted and the DPA d_2-**4** remained high, until the final time point at which the DPA d_2-**4** pool in the embryos was less than 10 ng/mg fr. wt. Throughout the experiment, the level of supplied ABA d_2-**1** in the embryos was relatively constant (Fig. 5b). Measurements of ABA d_2-**1** alone would not reflect the uptake and metabolism of the supplied hormone.

The initial concentration of the supplied labile 8′-OH ABA d_2-**2** was 13 μM in the culture medium, and unlike the (+)-ABA d_2-**1** experiment, at 6 h very little compound is taken up or metabolized (Fig. 4c). Using HPLC analysis, the equilibrium between 8′-OH ABA d_2-**2** and PA d_2-**3** was monitored over the time of the experiment. At 6 h most of the supplied 8′-OH ABA d_2-**2** remained open, but by 24 h, the concentration of the open form had decreased to one fifth and the level of PA d_2-**3** rose to 10 μM. Analysis of the embryo tissue supplied with 8′-OH ABA d_2-**2** demonstrates that pools of PA d_2-**3** do not accumulate (Fig. 6b), in contrast to the tissues supplied with ABA d_2-**1** (Fig. 5b). The accumulation of DPA d_2-**4** in the embryos in both ABA d_2-**1** (Fig. 5b) and 8′-OH ABA d_2-**2** treatments (Fig. 6b) are similar. Significant accumulation of DPA d_2-**4**, for the 8′-OH ABA d_2-**2** treatment, in the medium is seen only at 120 h (Fig. 6a).

For 7′-OH ABA d_2-**6** treatments, the levels of the deuterated compound in the medium remained high, with 50% persisting after the first 24 h (Figure S2 in supplemental material). A comparison of the HPLC and HPLC-MS/MS results of the media samples indicated

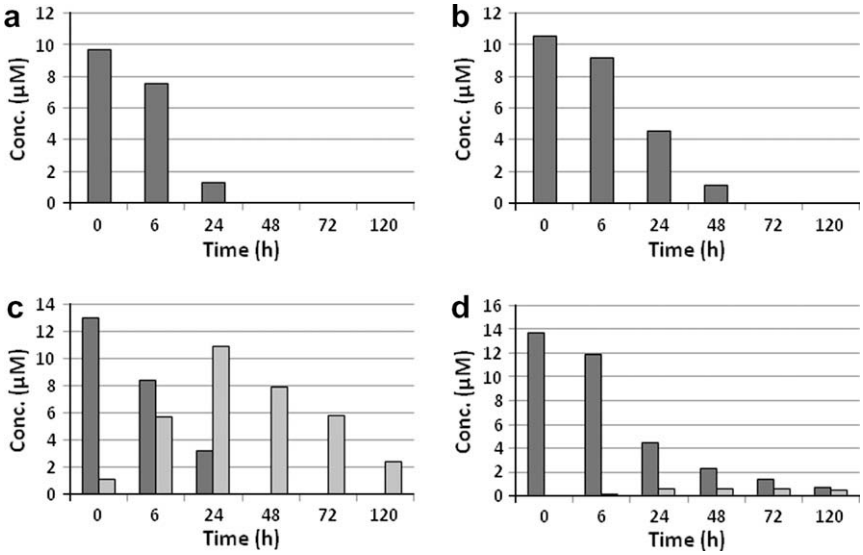

Fig. 4. Persistence of the supplied (a) ABA d_2-**1**, (b) 7′-OH ABA d_2-**2**, (c) 8′-OH ABA d_2-**2** (dark grey) and its closed form PA d_2-**3** (light grey) and (d) 9′-OH ABA d_2-7 (dark grey) and its closed form neoPA d_2-**8** (light grey), in the medium of cultured early cotyledonary stage microspore-derived embryos of *B. napus* cv *Hero*, as determined by HPLC. The quantitative analysis was performed while the experiment was on going to minimize changes to the equilibrium between open and closed forms, as described in the Experimental.

that the 7′-OH ABA d_2-**6** partially degraded under the storage conditions, so the levels of 7′-OH ABA d_2-**6** appearing in the embryos are likely lower than originally produced. No catabolites of 7′-OH ABA **6** are yet known, and no ABA **1** catabolites were observed in the MD embryos. The data suggest that uptake is similar to that of 8′-OH ABA **2**, with a lag at 6 h, before levels increase at 24 h.

In the case of treatment with 9′-OH ABA d_2-**7**, the equilibrium favors the open form, resulting in greater persistence of 9′-OH ABA d_2-**7** in the culture medium (Fig. 4d). By HPLC, only at 24 h, does any significant pool (10%) of *neo*PA d_2-**8** accumulate in the medium (Figure S3 in supplemental material). In unpublished work, it has been observed that on storage in the frozen state the equilibrium slowly shifts to the closed form. The shift is observed to a limited extent in the HPLC–MS/MS analysis of the medium, but the open form is still predominant and the levels are high, showing that degradation of the compounds is minimal. Analysis of the deuterated compounds in the embryo extract show that the predominant form is the closed form, indicating that possibly, the open form is poorly taken up into the embryos, and that the closed form *neo*PA **8** is preferentially taken up and then equilibrates. No other 9′-OH ABA **7** catabolites are known, and no ABA **1** catabolites were observed.

*2.3. Effect of ABA **1** and hydroxylated catabolites on oil content and oleosin gene expression in MD embryos*

An analysis of the oil content throughout the treatment period is shown in Fig. 7. At each incubation time, treatment with hormones enhanced oil accumulation compared to the corresponding controls. The oil accumulation performance in the presence of 8′-OH ABA **2**, comparable to ABA **1**, was similar to previous results reported for this "open" hydroxy form of the hormone in *B. napus* cv Reston MD embryos (Zou et al., 1995). Similar increases in oil accumulation observed in the present study with ABA **1**, 7′-OH ABA **6** and 9′-OH ABA **7** indicate that the metabolites have comparable biological activity with respect to oil synthesis.

To assess the effects of the hormones at the gene expression level, northern analyses were performed to measure the transcript levels of *Oleosin*, an ABA-inducible gene encoding an oil body protein (Zou et al., 1995). As shown in Fig. 8, treatment with ABA **1** and all hydroxylated metabolites resulted in increased levels of *Oleosin* transcripts. Higher steady-state levels of *Oleosin* transcript were detectable as early as 6 h following treatment with the hormones and reached a maximum at 24 h before decreasing at 48 h. Comparisons between different hormone treatments at specific time points indicate that ABA **1** and 9′-OH ABA **7** were more effective at inducing *Oleosin* expression than 7′-OH ABA **6** and 8′-OH ABA **2** at 6, 72 and 120 h (Fig. 8). At 24 and 48 h, 7′-OH ABA **6** appears to be as effective as ABA **1** and 9′-OH ABA **7**, while 8′-OH ABA **2** is weaker at all time points, with the possible exception of 24 h.

*2.4. Effect of ABA **1** and hydroxylated catabolites on FAE1 gene expression and VLCMFA content*

To further compare the effects of ABA **1** and the hydroxylated compounds on lipid bioassembly, we examined changes in the expression of the *FAE1* gene encoding KCS, which is the rate-limiting enzyme in the biosynthesis of VLCMFAs such as erucic and eicosenoic acids. The transcript for *FAE1* was induced strongly at 24 h for treatments with ABA **1** and 7′- and 9′- hydroxy ABA **6** and **7**, compared to the control and the 6 h time point which showed no significant induction (Fig. 9). By 24 h, there was a strong effect elicited by ABA **1**, and especially, 7′-OH ABA **6** and 9′-OH ABA **7**, and this induction remained strong throughout the incubation period. The response to 8′-OH ABA **2** was not significantly different from controls.

ABA **1** and the 7′- and 9′-hydroxylated metabolites **6** and **7** all exhibited an effect on VLCMFA biosynthesis, although the differences compared to controls only became highly significant after 5 d of incubation (Fig. 10). In a pattern similar to *FAE1* gene induction, the ABA **1**, 7′-OH ABA **6** and 9′-OH ABA **7** resulted in a doubling of VLCMFA proportions by 120 h, but the effect of the 8′-OH ABA **2** treatment was significantly lower than that observed with the other three hormone treatments, including ABA **1**, and only about 20% higher than the control treatment.

3. Discussion

*3.1. ABA **1** and catabolites in developing seeds of **B. napus***

The results show the presence of 7′-OH ABA **6** and 9′-OH ABA **7**, and significant pools of ABA **1**, as well as of 8′-oxidation ABA prod-

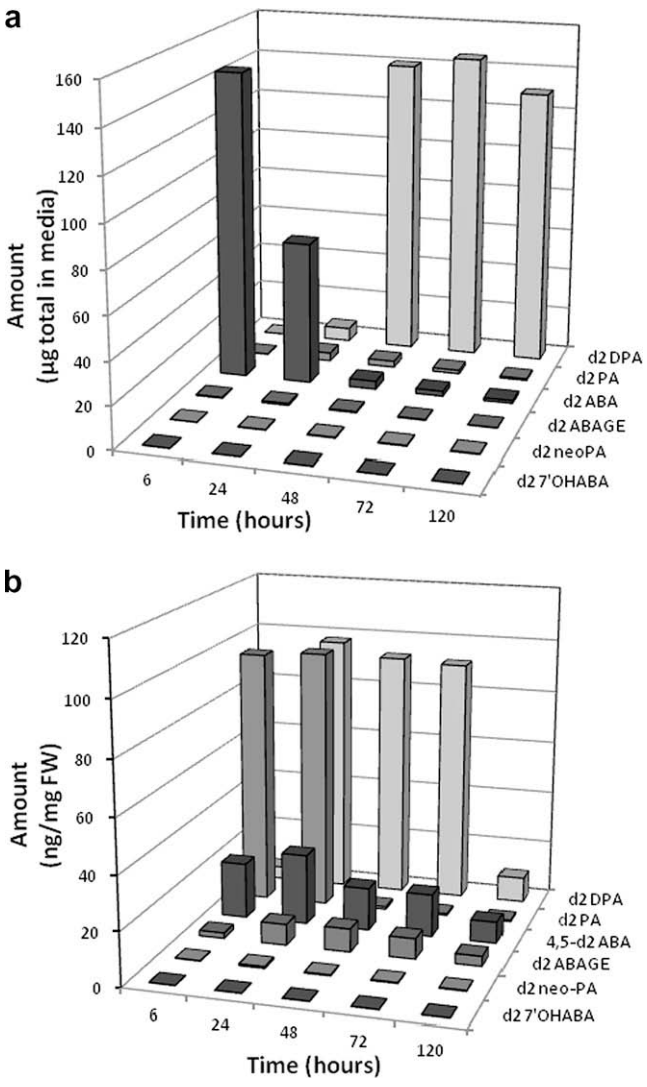

Fig. 5. Analysis of deuterated ABA compounds in (a) the medium and (b) embryos of early cotyledonary stage microspore-derived embryos of *B. napus* cv Hero treated with 4,5-d_2 ABA d_2-**1**. At each time point an aliquot of the medium was taken and embryos were harvested and samples were frozen. The samples were analyzed by HPLC-MS/MS as described in the Experimental.

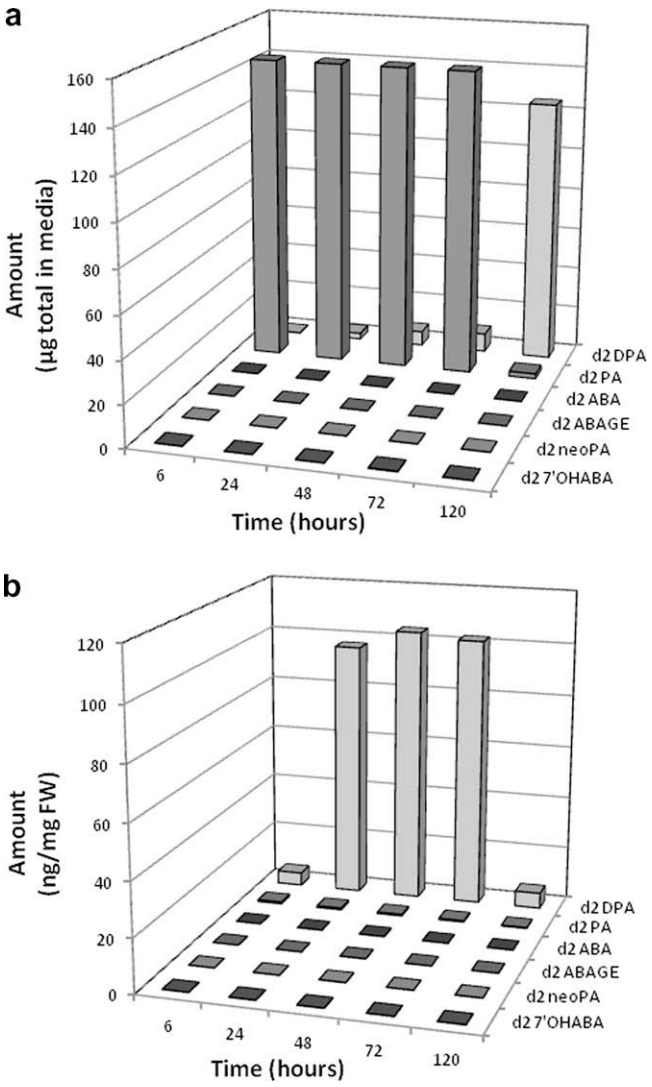

Fig. 6. Analysis of deuterated ABA, compounds in (a) the medium and (b) embryos of early cotyledonary stage microspore-derived embryos of *B. napus* cv Hero treated with 4,5-d_2 8′-OH ABA d_2-**2**. At each time point an aliquot of the medium was taken and embryos were harvested and samples were frozen. The samples were analyzed by HPLC–MS/MS as described in the Experimental.

ucts, present in developing *Brassica* seeds at 18–22 days post anthesis, when storage product accumulation is thought to occur. The developmental stage corresponds to the early cotyledonary MD embryo stage used throughout this study.

In general, there are two maxima in ABA **1** levels in seeds over the course of development (Karssen et al., 1983; Feurtado and Kermode, 2007). In Arabidopsis seeds, the first peak is greatest at 10 d after flowering (DAF), declining to a minimum 14 DAF and then increasing again. Storage product deposition is initiated at the same time as the levels of ABA **1** are reaching the first maximum. The source of the ABA **1** that regulates the process is thought to be of maternal and zygotic origin. Okamoto et al. (2006) quantified ABA **1**, PA **3** and DPA **4** and reported that the levels of DPA **4** had the same pattern as those of ABA **1**, with a maximum 10 DAF. An earlier study profiling ABA **1** catabolites in *B. napus* seed over development from mid maturation to mature seed reported that levels of ABA **1** peaked about 33 DAF with levels of DPA fourfold higher (Bonham-Smith et al., 2006). The catabolites ABA GE **9**, 7′-OH ABA **6**, 9′-OH ABA **7** or *neo*PA **8** were all found at lower but significant levels. Over seed maturation, the levels of ABA **1** and oxidized products decreased.

There have been few if any reports of direct measurement of ABA GE **9** in developing seeds. The ABA GE **9** may be of maternal origin and provide a source of stored ABA **1** for the developing seed (Lee et al., 2006). The endogenous levels of the hydroxylated catabolites were found to be relatively low (Fig. 2). The proportions of ABA **1** to PA **3** are similar in developing Arabidopsis and *Brassica* seeds, with the oxidized form about 10% of the ABA **1** level. However, the DPA **4** levels are at least as high as that of ABA **1**, indicating that oxidation through the 8′-hydroxylation route is a prominent pathway. As the catabolites of 7′-OH ABA **6** and *neo*PA **8** have not yet been identified, it is possible that these pathways are also significant. Okamoto et al. (2006) noted the 8′-catabolic pathway appeared to be the most important in Arabidopsis, as endogenous ABA **1** levels in *cyp707a* mutants were high.

3.2. Persistence and metabolism of ABA *1* and catabolites

The pools of ABA **1** and catabolites, observed by mass spectrometric analysis, in the control treatment embryos indicate that there is a very low level of ABA **1** metabolism occurring naturally within the MD embryos. In all cases, the levels of natural ABA **1**

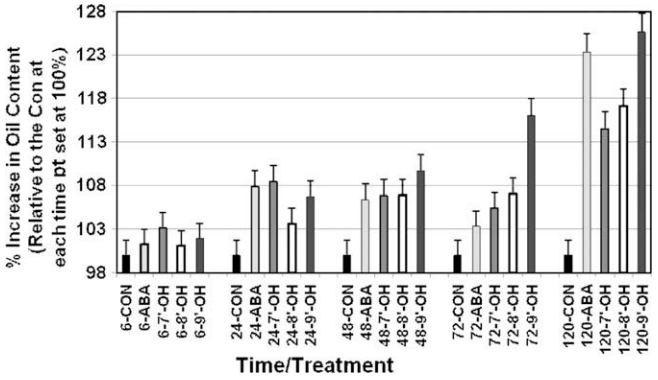

Fig. 7. Relative effects of ABA **1**, 7′-OH ABA 8′-OH ABA **2** and 9′-OH ABA **7** on oil accumulation in early cotyledonary stage microspore-derived embryos of *B. napus* cv Hero. Early cotyledonary stage embryos were treated with 10 μM ABA d_2-**1**, 7′-OH ABA d_2-**6**, 8′-OH ABA d_2-**2** or 9′-OH ABA d_2-**7** or 0.1% ethanol without hormone (Control) for 6, 24, 48, 72 and 120 h. After each time point, embryos were harvested and analyzed for oil content as described in Experimental. Percent increase in oil content is reported relative to control value set at 100% for each time point ± S.E.. The total oil content (% of dry wt) for the Control MD embryos at 6, 24, 48, 72 and 120 h were 18%, 17.5%, 17.3%, 17.0% and 17.0%, respectively. All hormone treatments were significantly elevated at all time points from 24–120 h.

compounds in the treated and control media were similar and below 0.1 μg in the total medium sample, indicating that there was no significant production and secretion of endogenous ABA **1** and catabolites into the culture medium (Supplemental data). This finding is in contrast to the case of ABA **1** supplied to *Marsilea* plants (Lin et al., 2005) where addition of deuterated (+)-ABA d_2-**1** was found to stimulate ABA **1** biosynthesis, resulting in greatly increased levels of unlabeled DPA **4** accumulating in the plants. The present results suggest that the observed biological activity of the supplied compounds was likely due to the chemical supplied or a metabolite of the treatment compound.

The results from the analysis of the medium and the embryos treated with ABA d_2-**1** suggest that uptake of ABA d_2-**1** is facile, and there is no observed build up of PA d_2-**3** in the culture medium (Fig. 5). It appears that hydroxylation of ABA d_2-**1** to 8′-OH ABA d_2-**2** is rapid and that the reduction of PA d_2-**3** to DPA d_2-**4** is even more rapid. The DPA d_2-**4** produced appears to be exported to the medium, and/or further metabolized. A small proportion of the supplied ABA d_2-**1** is glucosylated, and no significant accumulation of either 7′-OH ABA d_2-**6** or 9′-hydroxylation d_2-**7** products is found in embryos or media.

In the MD embryos treated with labeled 8′-OH ABA d_2-**2**, the pool of labeled PA/8′-OH ABA d_2-**2**/d_2-**3** was very small (Fig. 6b). By 24 h, the embryos contained high levels of DPA d_2-**4**, with no PA d_2-**3** observed. This could indicate that the 8′-OH ABA **2** is poorly taken up but requires cyclization to the less polar PA **3** for uptake which does not accumulate, but is rapidly reduced to DPA **4**. There appears to be a lag time between the accumulation of DPA d_2-**4** in the embryos and the export of the metabolite to the medium. It is interesting to compare the profiles of DPA d_2-**4** appearing in the medium resulting from either the ABA d_2-**1** treatment (Fig. 5a) or the 8′-OH ABA d_2-**2** treatment (Fig. 6b). At 48 h, DPA d_2-**4** levels are very high in the medium from the ABA d_2-**1** treatment, whereas high levels of DPA in the 8′-OH ABA d_2-**2** treatment are only observed at 120 h. Part of the explanation likely comes from poor uptake of the supplied 8′-OH ABA d_2-**2** and PA d_2-**3**. Further oxidation products of 8′-OH ABA **2** and PA **3** have been reported, but were not analyzed in these experiments (Zaharia et al., 2004).

Taken together the metabolism data are consistent with the scheme shown in Fig. 11. While this scheme applies only to the model MD embryo system it may be relevant to uptake, transport and metabolism of ABA **1** compounds in *Brassica* seeds or other plant

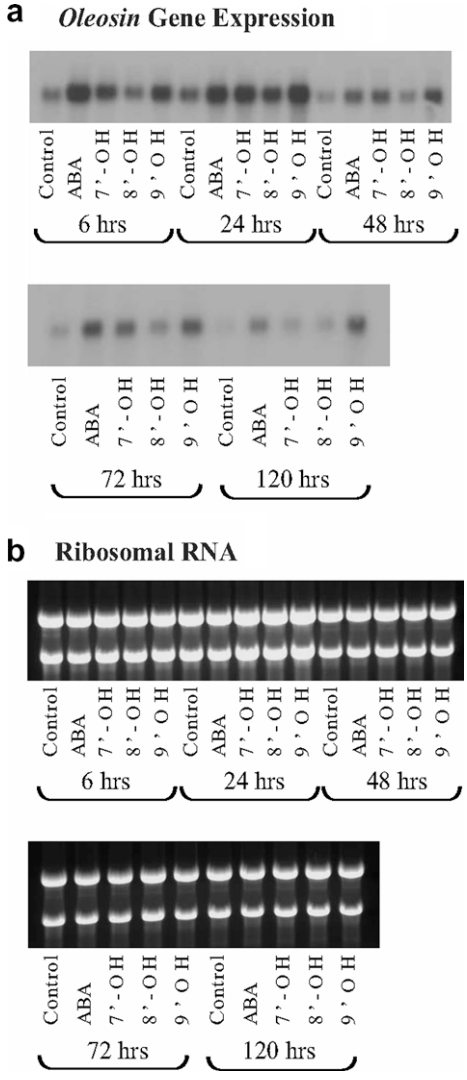

Fig. 8. Effects of ABA **1**, 7′-OH ABA **6**, 8′-OH ABA **2** and 9′-OH ABA **7** on induction of *Oleosin* gene expression in early cotyledonary stage microspore-derived embryos of *B. napus* cv Hero. Early cotyledonary stage embryos were treated with 10 μM ABA d_2-**1**, 7′-OH ABA d_2-**6**, 8′-OH ABA d_2-**2** or 9′-OH ABA d_2-**7** or 0.1% ethanol without hormone (Control) for 6, 24, 48, 72 and 120 h. After each time point, embryos were harvested and analyzed for *Oleosin* gene induction as described in Experimental. A, Relative expression of *Oleosin* gene transcript; B, relative equal loading of total RNA on the gel as determined by comparative intensities of the two characteristic ribosomal RNA bands. Similar results were obtained in an independent experiment with RNA collected from different preparations of MD embryos.

cells. Uptake of less polar compounds ABA **1**, PA **3** and *neo*PA **8** may be more facile than uptake of 7′-, 8′-, and 9′- OH ABA **6**, **2** and **9**. PA **3** is rapidly metabolized to DPA **4** and is not exported from the embryos. We had no way to assess the equilibrium between open and closed forms within the embryos, and the contribution of each to the activity observed. There is a delay in export of DPA **4** from the embryos, but then a massive release of the compound occurs. ABA GE **9** appears not to be exported, but retained in the embryos.

*3.3. Effect of ABA **1** and hydroxylated catabolites on oil content and oleosin gene expression in MD embryos*

In the present study supplied ABA d_2-**1**, 7′-OH ABA d_2-**6** and 9′-OH ABA d_2-**7** all resulted in significant increases in oil accumulation indicating that the metabolites have comparable biological activity with respect to oil synthesis. The oil accumulation performance of embryos supplied with 8′-OH ABA d_2-**2** was slightly lower than that

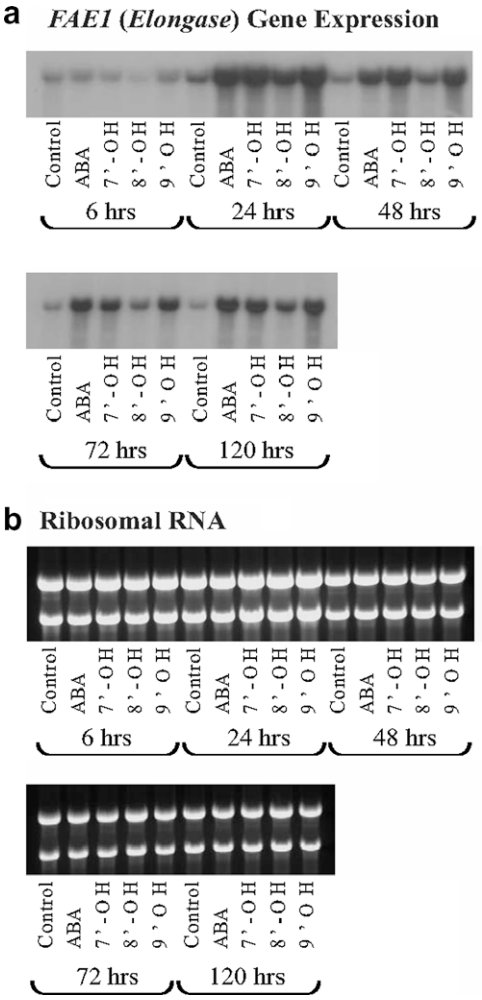

Fig. 9. Effects of ABA **1**, 7′-OH ABA **6**, 8′-OH ABA **2** and 9′-OH ABA **7** on induction of 3-keto-acyl-CoA synthetase gene (*FAE1*) expression in early cotyledonary stage microspore-derived embryos of *B. napus* cv Hero. Early cotyledonary stage embryos were treated with 10 μM ABA d_2-**1**, 7′-OH ABA d_2-**6**, 8′-OH ABA d_2-**2** or 9′-OH ABA d_2-**7** or 0.1% ethanol without hormone (Control) for 6, 24, 48, 72 and 120 h. After each time point, embryos were harvested and analyzed for *FAE1* gene induction as described in Experimental. A, Relative expression of *FAE1* gene transcript; B, relative equal loading of total RNA on the gel as determined by comparative intensities of the two characteristic ribosomal RNA bands. Similar results were obtained in an independent experiment with RNA collected from different preparations of MD embryos.

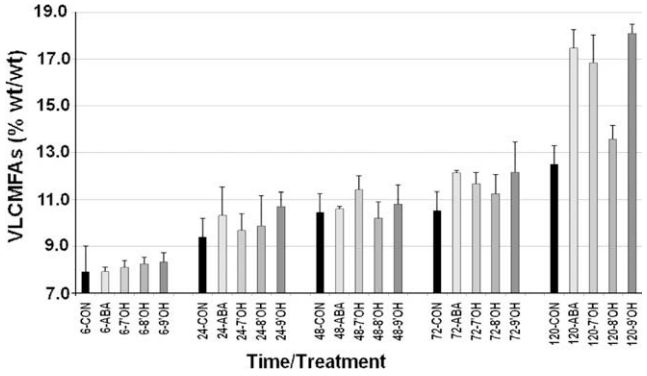

Fig. 10. Relative effects of ABA **1**, 7′-OH ABA **6**, 8′-OH ABA **2** and 9′-OH ABA **7** treatments on the proportions of very long chain mono-unsaturated fatty acids (VLCMFAs; 20:1, 22:1 and 24:1) in microspore-derived embryos of *B. napus* cv Hero. Early cotyledonary stage embryos were treated with 10 μM ABA d_2-**1**, 7′-OH ABA d_2-**6**, 8′-OH ABA d_2-**2** or 9′-OH ABA d_2-**7** or 0.1% ethanol without hormone (Control) for 6, 24, 48, 72 and 120 h. After each time point, embryos were harvested and analyzed for fatty acid composition as described in Experimental. The total proportion of VLCMFAs (% wt/wt) in the total lipid extract ± S.E. is reported for each treatment/time point. The hormone enhancement effects were consistently significant by the 120 h time point.

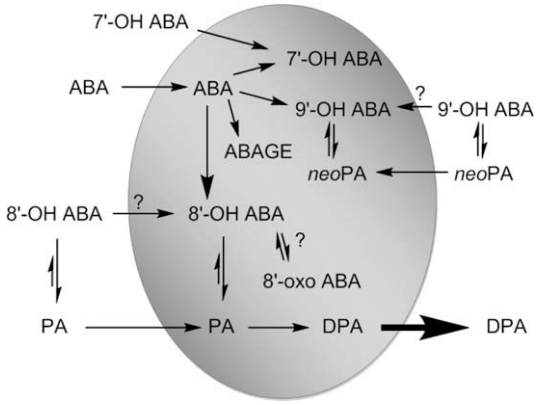

Fig. 11. Model of uptake and metabolism of ABA **1** and catabolites in MD embryos.

of supplied ABA d_2-**1**, as had been found previously for this "open" hydroxy form of the hormone in *B. napus* cv Reston MD embryos (Zou et al., 1995). Likely this is due to the shift towards PA d_2-**3**.

The metabolite 8′-OH ABA **2** was previously shown to induce the lipid-related genes *Oleosin* and *FAD3* in MD embryos (Zou et al., 1995). The present data indicate that 7′-OH ABA **6** and 9′-OH ABA **7** are at least as effective, if not more effective, than 8′-OH ABA **2** at inducing lipid-related gene expression. This gene expression induction likely accounts, at least in part, for the ability of these metabolites to enhance oil accumulation in MD embryos. Interestingly, the levels of the transcript for diacylglycerol acyltransferase 1 (*DGAT1*) which encodes a rate-limiting step in TAG assembly (Zou et al., 1999; Jako et al., 2001) were unaffected by ABA **1** or ABA catabolites in MD embryos (unpublished).

*3.4. Effect of ABA **1** and hydroxylated catabolites on FAE1 gene expression and VLCMFA content*

ABA **1** and the various hydroxylated metabolites all exhibited an effect on VLCMFA biosynthesis, although the differences compared to controls only became highly significant after 5 d of incubation (Fig. 10). In a pattern similar to *FAE1* gene induction, the ABA d_2-**1**, 7′-OH ABA d_2-**6** and 9′-OH ABA d_2-**7** resulted in a doubling of VLCMFA proportions by 120 h, but the effect of the 8′-OH ABA d_2-**2** treatment was significantly lower than that observed with the other three hormone treatments, including ABA d_2-**1**, and only about 20% higher than the control treatment. This finding differs in degree with that previously observed in studies where 8′-OH ABA **2** (Zou et al., 1995) were supplied to *B. napus* cv Reston embryos; in those studies, the erucic acid proportions were increased by 60–100% over controls. The reason for this difference is not clear. It is possible that this is due to the difference in HEAR cultivar utilized in the current work (Hero) *vs* former studies (Reston). Another variable is the rate of closure of 8′-OH ABA **2** to PA **3** in different experiments. To address this issue we had synthesized analogs of 8′- and 9′-OH ABA **2** and **7** that could not cyclize to PA **3**- and *neo*PA **8**-like compounds (Nyangulu et al., 2006). In two assays for ABA **1** activity, a maize cell growth inhibition assay and also an Arabidopsis seed germination inhibition assay, the analog resembling 9′-OH ABA **7** was equal to or more potent than ABA **1** and the analog mimicking 8′-OH ABA **2**. These results are consistent with the findings in the present study.

4. Conclusions

In these and other experiments in which ABA **1** is supplied to plant tissues, it is difficult to determine if ABA **1** is acting on its own or is converted to an active metabolite which is the biologically active form. The levels of gene expression observed are the combined result of the activity of the compound and the level in the tissue, and the 8′-OH ABA **2** is the most labile, isomerizing readily to PA **3**. In the normal pattern of ABA **1** catabolism, nearly all ABA **1** is oxidized to 8′-OH ABA **2**. We had previously shown that 8′-OH ABA participated in an oxidation reduction cycle involving ABA 8′-aldehyde, which could effectively prolong the life time of 8′-OH ABA **2**. It is possible that effects observed with 8′-OH ABA **2** treatments do reflect the activity of this transient metabolite.

No increase in endogenous ABA **1** biosynthesis was observed in the MD embryos treated with the hydroxylated ABA compounds. Thus, the implications for determining the cause of the biological results, i.e. that supplied 7′-OH ABA d_2-**6** as well as the 8′-, and 9′-OH ABA d_2-**2** and d_2-**7** all induce lipid-related genes and accumulation of TAGs is that the supplied hydroxy-ABAs, or their metabolites, are all effectors of the storage lipid response in their own right. The roles of these compounds in other ABA-regulated processes require further elucidation. Despite the metabolic evidence that the hydroxyl ABAs were taken up less rapidly, treatments with these compounds were capable of stimulating the oil deposition process, with 9′-OH ABA **7** showing a very strong induction capability. It will be possible to establish the importance of the hydroxylated compounds with more certainty once the enzymes that oxidize ABA **1** at the 7′- and 9′-methyl groups have been identified and genetic studies performed. When the relevant ABA receptor has been identified it will be possible to perform in depth binding studies with ABA **1** and its metabolites. Our present study shows that the binding pocket of the biomolecules recognizing ABA **1** or hydroxy ABA can accommodate the additional hydroxyl groups on the methyl groups of the ABA ring. Structural analogs of ABA, including stable analogs of 8′- and 9′-hydroxy ABA, should be useful for dissecting ABA receptor responses (Nyangulu et al., 2006).

5. Experimental

5.1. Preparation of deuterated ABA d_2-*1* and hydroxylated catabolites

The ABA **1** and ABA hydroxylated compounds used as feeding substrates were deuterium labeled. (+)-4,5-d_2-ABA d_2-**1** was prepared as previously published (Abrams et al., 2003). The deuterium labels for (+)-7′,7′-d_2-7′-hydroxy ABA d_2-**6** (PBI-703) and (+)-9′,9′-d_2-9′-hydroxy ABA d_2-**7** (PBI-705) were incorporated by reduction of 3-methylsalicylic acid with $LiAlD_4$ to give d_2-3-methylsalicyl alcohol. This intermediate was further elaborated to give the ABA metabolites using methods previously described (Nelson et al., 1991; Rose et al., 1996). The full syntheses of d_2-7′-OH ABA **6** and d_2-9′-OH ABA **9** will be reported elsewhere (Abrams et al., in preparation). (+)-4,5-d_2-8′-hydroxy ABA d_2-**2** was prepared as described below.

5.2. Preparation of (+)-4,5-d_2-8′-hydroxy ABA d_2-*2*

In a small round bottom flask or glass vial, to a solution of (−)-4,5-d_2-PA d_2-**3** (0.5 mg, 2 μmol, from (+)-4,5-d_2-ABA d_2-**1** biotransformation by cultured Maize cells (Balsevich et al., 1994)) in glacial AcoH (1 mL), boric acid (0.25 mg, 4 μmol) was added. The reaction was set in an oil bath at 100 °C, under an argon atmosphere. After stirring for 1 h in the dark, the mixture was allowed to cool, and the completion of the reaction was monitored by HPLC. A small portion (10 μL) was diluted with a mixture of H_2O:acetonitrile (250 μL, 1:1, v/v), then analyzed by HPLC. The reaction mixture was further dried under reduced pressure. To prevent the closure of 8′-OH ABA d_2-**2** to PA d_2-**3**, the compound was used within the next hour. The compound was reconstituted in 1.0 mL water:ethanol (3:1, v/v) and rapidly transferred by pipette to the culture medium of the MD embryos. The final concentration in the media was 10 μM.

5.3. Microspore culture and hormone treatments

Seed of *B. napus* L cv Hero, a high erucic cultivar, was obtained from Dr. P.B.E. McVetty at the University of Manitoba. Plants were grown in a controlled environment growth chamber and microspores were isolated and cultured according to the methods described previously (Taylor et al., 1991; Holbrook et al., 1992). The NLN culture medium was modified from that previously described (Lichter, 1982). Media contained 13% sucrose, 0.83 mg l^{-1} KI but no potato extract or hormones. The pH of the medium was adjusted to 5.8. Cultures were incubated at 32 °C for 72 h then maintained at 24 °C. MD embryo preparations (constituting 15–20 g fr. wt of embryos) enriched in the early cotyledonary stage were obtained by filtration through a sterile 0.2 μm mesh filter and replated at a density of 0.20–0.25 g fr. wt in 10 mL of medium in 100 × 10 mm Petri plates.

After a 24 h equilibration period, embryos were supplemented with medium containing either (+)-ABA d_2-**1**, 7′-OH ABA d_2-**6**, 8′-OH ABA d_2-**2** or 9′-OH ABA d_2-**7** at a concentration of 10 μM in 0.1% (v/v) EtOH. Control treatments contained medium supplemented with 0.1% (v/v) EtOH only. Cultures were maintained in the dark at 25 °C with gentle shaking on a platform shaker at 50 rpm. Fifteen plates of embryos were treated for each hormone. Three plates of each treatment, representing several thousand embryos, were harvested after 0, 6, 24, 48, 72 and 120 h by suction filtration. The medium was saved for hormone/metabolite analysis. The fresh weight of each embryo sample was recorded, and embryos immediately frozen in liquid nitrogen and stored at −70 °C until analyzed. Portions of the embryo samples of a defined fr. wt were then desiccated at 100 °C for dry weight determination. Other embryo batches from each treatment and time point were used to measure total lipid content and acyl composition, or for hormone analysis.

5.4. Extraction of total lipids from MD embryos and analysis of fatty acid composition and oil content

Embryo samples were weighed and transferred to a cooled mortar and ground in 2 mL *iso*-PrOH:CH_2Cl_2 (2:1), the mixture was transferred to a test tube; to this was added the above solvent (1 mL) and 0.9% NaCl (1 mL) and vortexed. 2 mL CH_2Cl_2 was added, the mixture re-vortexed and centrifuged at 2500 rpm for 3 min. The CH_2Cl_2 layer was removed, the extraction repeated and the CH_2Cl_2 layers combined. CH_2Cl_2: benzene: methanol (1:1:1) (1 mL) was added and then the sample evaporated to dryness. The dried sample was resuspended in $CHCl_3$ (1 mL) to give the total lipid extract (TLE). Determination of oil content and acyl composition were performed as described previously (Jako et al., 2001). While trends were similar in each of three experiments, there was considerable variation in the absolute values for these oil parameters. Thus, the results of one representative experiment are reported in the Figures and oil analyses represent the average of three determinations.

5.5. Northern analysis

Total RNA from MD embryo plant material was isolated as previously described (Wang and Vodkin, 1994). Five μg of RNA was

fractionated on a 1.4% formaldehyde-agarose gel and the gels were then stained with ethidium bromide to ensure that all lanes had been loaded equally (Sambrook et al., 1989). The RNA was subsequently transferred to a GeneScreen + Hybridization Transfer Membrane (NEN Life Sciences Products, Inc., Boston, MA), UV cross-linked and hybridized at 68 °C in QuickHyb solution (Stratagene, LaJolla, CA) with α-^{32}P-dCTP radiolabeled probes (random primers, Invitrogen, Burlington, ON) consisting of fragments from the coding regions of the *B. napus Oleosin* (Genbank accession EE400567) or *FAE1* (Genbank accession U50771) genes. Following hybridization, membranes were washed twice at room temperature in 2 × SSC, 1% SDS for 15 min and once in 0.1 × SSC, 0.1% SDS at 65 °C for 30 min, and exposed on X-ray film (Agfa, CanMed HealthCare, Edmonton, AB).

5.6. Growth of plant material for analysis of ABA metabolites in seed

B. napus cv Quantum seeds were germinated on moist filter paper in the dark. Seedlings were transferred to soil (Readi-Earth) in environmentally controlled cabinets with a 16 h day (22 °C; 175 $\mu Em^{-2}\ s^{-1}$)/8 h night (17 °C) photoperiod and watered daily with a 1:10 dilution of 20:20:20 (nitrogen:phosphorous:potassium) fertilizer. Flowers on the primary and secondary inflorescences were self-pollinated at anthesis and tagged. Seeds were dissected from siliques on dry ice, weighed, and frozen in liquid N_2.

5.7. Metabolism studies of ABA d_2-**1** and catabolites

An aliquot (250 μL) of the medium was taken and analyzed by HPLC for each treatment at the time points of 0, 6, 24, 72 and 120 h. Samples were placed in a syringeless filter device (Whatman Mini-UniPrep, PTFE, 0.45 μm pore size), diluted with CH_3CN (250 μL) and injected (10 μL) on the HPLC as quickly as possible. The detector (262 nm) was calibrated against standards of known concentration. The calibration curve for 9′-OH ABA d_2-**7** was used for 8′-OH ABA d_2-**2**. Analytical HPLC analysis was carried out with an Agilent HPLC 1100 series (Agilent, Palo Alto, CA) equipped with a quaternary pump, automatic injector, diode array detector (wavelength range 190–950 nm), degasser, and a Suplcosil LC-18 column (4.6 mm i.d. × 33 mm, 3 μm particle size silica), equipped with a guard column. The retention time (R_t) is reported for a gradient elution (0.1% HOAc in H_2O/0.1% HOAc in CH_3CN, 95:5 for 10 min, ramped to 85:15 over 5 min) at a flow rate of 1.5 mL min^{-1}. Rts were: ABA **1** 17.6 min, 7′-OH ABA **6** 14.4 min, 8′-OH ABA **2** 13.5 min, PA **3** 11.6 min, 9′-OH ABA **7** 11.8 min and *neo*PA **8** 15.2 min.

Embryo samples were frozen in liquid N_2, stored at −20 °C and then freeze-dried, and 50 mg samples were homogenized. To each sample 40 μL internal standard in a mixture of H_2O:CH_3CN (1:1, v/v) containing d_4-ABA (PBI 636), d_3-PA (PBI 241), d_3-DPA (PBI 674), d_4-7′-OH ABA (PBI 667), d_3-*neo*PA (PBI 680) and d_5-ABA GE (PBI 671) (each in the concentration of 0.5 ng/μL) was added. Further, the extraction solvent (1 mL mixture of *iso*-PrOH:glacial AcoH 99:1, v/v) and a small magnetic stir bar were added to each sample vial. After 12 h of stirring in the dark at room temperature, the samples were centrifuged at 1500*g* for 10 min, the supernatant was transferred to a vial, and a second portion of 1 mL extraction solvent mixture was added. After stirring in the dark for another 4 h, the samples were centrifuged and the supernatant was combined with the previous one. The organic extract was dried under reduced pressure, then re-dissolved in 100 μL MeOH:glacial AcOH (99:1, v/v) followed by 900 μL of aqueous 1% glacial AcOH. This mixture was further cleaned on an OASIS cartridge (Waters OASIS extraction cartridge HLB 1 cc). The fraction containing ABA and ABA metabolites was eluted with 1.5 mL MeOH:H_2O:glacial AcoH (80:19:1, v/v/v) and then was evaporated to dryness. The final residue was dissolved in a 200 μL (2 × 100 μL portions) mixture of H_2O:CH_3CN (80:20, v/v) with 0.07% glacial AcoH containing 20 ng d_6-ABA **1** (PBI 322, used as an external standard), and then subjected to mass spectrometry/LC-ES-MS-MS analysis (Feurtado et al., 2004; Ross et al., 2004).

Table 1
Summary of compounds (retention times for HPLC/MS, MRM transitions used for quantification)

Compounds analyzed[a]		Transitions[b]		
Name	Abbreviation	RT (min)	ES-precursor ion (*m/z*)	ES-product ion (*m/z*)
Endogenous				
Abscisic acid	ABA	13.5	263	153
Dihydrophaseic acid	DPA	4.4	281	171
Abscisic acid glucose ester	ABAGE	8.8	425	263
Phaseic acid	PA	9.4	279	205
7′-Hydroxyabscisic acid	7′OH-ABA	10.6	279	151
neo-Phaseic acid	*neo*-PA	11.7	279	205
4,5-d_2 ABA treatment				
Abscisic acid	4,5-d_2 ABA	13.4	265	153
Dihydrophaseic acid	4,5-d_2 DPA	4.4	283	172
Phaseic acid	4,5-d_2 PA	9.4	281	207
4,5-d_2 8′OH-ABA treatment				
Abscisic acid	4,5-d_2 ABA	13.4	265	153
Dihydrophaseic acid	4,5-d_2 DPA	4.4	283	172
8′-Hydroxyabscisic acid	4,5-d_2 8′OH-ABA	9.9	281	207
Phaseic acid	4,5-d_2 PA	9.6	281	207
7′,7′-d_2 7′OH-ABA treatment				
7′-hydroxyabscisic acid	7′,7′-d_2 7′OH ABA	10.3	281	153
9′,9′-d_2 9′OH-ABA treatment				
9′-Hydroxyabscisic acid	9′,9′-d_2 9′OH-ABA	8.5	281	205
neo-Phaseic acid	9′,9′-d_2 *neo*-PA	11.4	281	205
Internal standards				
Abscisic acid	5,8′,8′,8′-d_4 ABA	13.4	267	156
Dihydrophaseic acid	7′,7′,7′-d_3 DPA	4.4	284	174
Abscisic acid glucose ester	4,5,8′,8′,8′-d_5 ABAGE	8.7	430	268
Phaseic acid	7′,7′,7′-d_3 PA	9.4	282	142
7′-Hydroxyabscisic acid	5,8′,8′,8′-d_4 7′OH-ABA	10.5	283	154
neo-Phaseic acid	8′,8′,8′-d_3 *neo*-PA	11.7	282	208

[a] For any of the treatments with labeled ABA or ABA metabolites, both endogenous (non-labeled) hormones and labeled supplied compound and its metabolites were analyzed.

[b] MRM of ABA and metabolites was based on the transition from the deprotonated molecular ion ($[M-H]^-$, precursor) to the predominant fragment ion (product). All compounds were analyzed in the negative ion mode (Feurtado et al., 2004).

The media in which the embryos were grown was also analyzed. Thus, to the medium sample (1 mL), 40 μL an internal standard mixture of d_4-ABA **1** (PBI 636), d_3-PA **3** (PBI 241), d_3-DPA **3** (PBI 674), d_4-7′-OH ABA **6** (PBI 667), d_3-*neo*PA (PBI 680) and d_5-ABA GE **9** (PBI 671) (each in the concentration of 0.5 ng/μL) in H_2O:CH_3CN (1:1, v/v) was added. The mixture was vortexed, and then further cleaned on an OASIS cartridge (Waters OASIS extraction cartridge HLB 1 mL). The fraction containing ABA **1** and ABA metabolites was eluted with 1.5 mL MeOH:H_2O:glacial AcoH (80:19:1, v/v/v) and then was evaporated to dryness. The dried media sample was further treated in the same manner as the embryo final residue, described above.

5.8. Liquid chromatography–mass spectrometry

Samples and standards were analyzed by HPLC–MS/MS using an Alliance™ 2695 HPLC system coupled to a Quattro™ Ultima™

quadrupole tandem mass spectrometer via a Z-spray™ electrospray ion source operating in the negative ionization mode (Waters Corporation, Beverly MA, USA). HPLC was performed using a C_{18} analytical column (Genesis™, 2.1 × 100 mm, 4-μm particle size; Jones Chromatograph, Lakewood CO, USA) and a narrow-bore C_8 guard column (Zorbax™ XDB-C8, 2.1 × 12.5 mm, 5-μm; Agilent, Santa Clara CA, USA). Separations were achieved using an initial solvent composition of 15.00:84.96:0.04% v/v CH_3CN/H_2O/AcOH and a flow rate of 200 μL/min. The CH_3CN content was increased linearly to 35.00:64.96:0.04% v/v CH_3CN/H_2O/AcOH during the first 10 min, then non-linearly to 40.00:59.96:0.04% v/v CH_3CN/H_2O/AcOH during the next 5 min, before flushing the column with 60.00:39.96:0.04% v/v CH_3CN/H_2O/AcOH for 3.2 min, and with 99.00:0.96:0.04% v/v CH_3CN/H_2O/AcOH at 350 μL/min for a further 1.3 min. The column was then re-equilibrated at the initial solvent flow rate and composition for 5 min before making the next sample injection. Injections were made using 10 μL of each 200 μL sample, with a total cycle time (injection to injection) of 28 min.

Analytes resolved by HPLC were detected and quantified by MS/MS using a scanning method multiple reaction monitoring (MRM), with a collision cell filled with argon gas, at a pressure of 1.3×10^{-3} mbar. The precursor-to-product ion MRM transitions for each analyte and internal standard are summarized in Table 1. Analytes were quantified by comparing the MRM chromatographic peak area determined for each analyte with that obtained for the corresponding internal standard (Table 1) using the QuanLynx™ software in MassLynx™ version 4.0 (Waters) with reference to calibration curves generated for each analyte (Feurtado et al., 2004; Ross et al., 2004). In cases where the labeled (d_2) ABA **1** or hydroxylated-ABA catabolite was unavailable to generate a calibration curve, the amount of deuterated metabolite in the sample was estimated using a calibration curve for the corresponding unlabeled compound, which was assumed to have a similar response factor to its labeled counterpart. Responses that were below the calibration range were recorded as being less than the limit of quantification (LOQ), while those that exceeded the calibration range were recorded as being greater than the maximum limit of quantification (actual limits not reported).

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2008.08.010.

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