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Metabolic and hormonal processes associated with the induction of secondary dormancy in *Brassica napus* seeds[1]

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Abstract: Polyethylene glycol treatment induces secondary seed dormancy in *Brassica napus* L. cultivar 'AC Excel' (ACE), but not in 'DH12075' (DH). Gene expression, metabolite profiles, and hormone profiles were obtained from seeds of both cultivars following polyethylene glycol 8000 treatment. ACE seeds were more transcriptionally active: 28 genes were up-regulated in both cultivars and 10 and 158 genes were specifically up-regulated in DH and ACE, respectively. Nontargeted metabolite analyses combined with gene expression analyses showed significant differences in lipid, sugar, and phenylpropanoid metabolism between the cultivars. Abscisic acid (ABA) levels were higher and many ABA-inducible genes were expressed more in ACE. An association of ABA with secondary dormancy was supported by the observation that secondary dormancy was induced by polyethylene glycol 8000 in *Arabidopsis* wild-type seeds, but was reduced in ABA-deficient and ABA-insensitive mutants. Therefore, secondary dormancy appears to be realized through an active ABA-related mechanism that may involve changes in primary and secondary metabolism.

Key words: canola, microarray, metabolite profiling, abscisic acid, polyethylene glycol, lipids, phenylpropanoid, dormancy.

Résumé : Un traitement avec le polyéthylène glycol (PEG) induit une dormance séminale secondaire chez le *Brassica napus* L. cultivar 'AC Excel' (ACE), mais non pas chez le cultivar 'DH12075' (DH). Les auteurs ont obtenu les profils de l'expression génétique, des métabolites et des hormones à partir de graines des deux cultivars suite à un traitement au PE 8000. Les graines ACE transcrivent plus activement avec 28 gènes surexprimés chez les deux cultivars et 10 et 158 gènes spécifiquement surexprimés chez le DH et le ACE, respectivement. L'analyse de métabolites non ciblés combinée avec les analyses de l'expression génétique montre des différences significatives dans le métabolisme des lipides, des sucres et du phénylpropanoïde entre les cultivars. On observe chez l'ACE des teneurs en acide abscissique (ABA) plus élevées et une plus forte expression de plusieurs gènes induits par l'ABA. Une association de l'ABA avec la dormance secondaire est supportée par l'observation que ;a dormance secondaire est induite par le PEG 8000 chez les graines de l'*Arabidopsis* de type sauvage, mais est réduite chez les mutants déficients en ABA ou insensible à l'ABA. Conséquemment, la dormance secondaire semble se réaliser par un mécanisme actif relié à l'ABA, qui peut impliquer des modifications dans les métabolismes primaire et secondaire.

Mots-clés : canola, microarray, profile métabolique, acide abscissique, polyéthylène glycol, lipides, phénylpropanoïde, dormance.

[Traduit par la Rédaction]

Introduction

Canola (*Brassica napus* L.: formerly known as oilseed rape) has become one of the most important cultivated oilseed crops worldwide. However, some improvements are required to maximize the efficiency of growth and harvesting of this crop. Yields are frequently reduced owing to seed shedding arising from preharvest pod shatter, and crop disturbance during harvesting (Price et al. 1996). In adverse conditions losses can reach 10 000 seeds·m^{-2} (Lutman 1993). These seeds remain viable in the soil for months or even years (Roller et al. 2002), and can act as a long-term source of "volunteer" plants. Erratic germination of volunteers may reduce quality in subsequent canola crops or act as a weed in other crop plants grown on the same land. In addition, cross pollination between volunteers from different genetically modified canola cultivars has the potential to cause technical problems with weed control, as a consequence of gene stacking.

The persistence of volunteer seed is affected by light and temperature (Pekrun et al. 1997*a*) and is related to the potential for the development of secondary seed dormancy. *Brassica napus* seeds exhibit little or no primary dormancy

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(Lutman 1993; Pekrun et al. 1998*b*; Momoh et al. 2002), but the induction of secondary dormancy is triggered in certain nondormant seeds by abiotic stresses such as light, moisture, anoxia, and temperature during seed storage or during germination (Hilhorst 1995, 1998; Pekrun et al. 1997*a*, 1998*a*; Toorop et al. 2005). *Brassica napus* exhibits large genotypic variations in its capacity for secondary dormancy, as reported by Pekrun et al. (1997*a*), Momoh et al. (2002) and Gruber et al. (2004). After inductive treatments, the proportion of dormant seeds ranges from almost zero up to 60% for winter genotypes and up to 85% for spring types. Within genotypes, variation occurs among seed lots and years of harvest. Osmotic stress is the most effective treatment for inducing secondary dormancy relative to other factors, such as altering oxygen levels and temperature, although induction varies considerably among and within genotypes (Pekrun et al. 1997*a*, 1997*b*). In the laboratory, secondary dormancy is conveniently induced by several weeks of treatment with polyethylene glycol (PEG) (Momoh et al. 2002; Gulden et al. 2004*b*). Recently, Gulden et al. (2004*b*) quantified the contributions of genotype, seed size, and environment to secondary dormancy potential in *B. napus*. The results suggested that genotype contributes from 44% to 82% of the total variation in secondary dormancy among 16 genotypes. Seed size contributes 21% of the variation and the effect of different harvest regimes contributes 0.1% to 4.5%.

In a previous study of gene expression during seed maturation, we compared the *B. napus* cultivar 'DH12075' (DH), which exhibits no secondary dormancy, with 'AC Excel' (ACE), in which secondary dormancy can be induced. We showed that many genes exhibit similar expression patterns in both cultivars and a majority of genes are down-regulated during maturation and dehydration. There are very few differences in gene expression among cultivars at the mature seed stage, but a significant number is apparent in the developmental transition from full-size embryo to mature seed. Most differences are apparent in the dehydration stage (Fei et al. 2007). These results suggested that the potential for secondary dormancy was determined during seed maturation. To better understand the responses of seeds to dormancy-inducing treatments, we profile differences in transcriptomes and metabolomes between dormant ACE seeds and DH seeds treated with PEG.

Material and methods

Plant materials

The growth conditions for *B. napus* DH and ACE were described previously (Fei et al. 2007).

Induction of secondary seed dormancy in *B. napus*

Mature seeds of DH were treated in 14.5 cm (diameter) Petri dishes lined with two layers of filter paper. Seeds (300) were incubated in each dish containing 22 mL of polyethylene glycol 8000 (PEG) solution with an initial osmotic potential of –1.5 MPa, in the dark, at 20 °C for 4 weeks (Pekrun et al. 1997*c*). The seeds were rinsed with distilled water (under green light) to remove PEG. The DH seeds, which cannot be induced into secondary dormancy, were put in liquid nitrogen immediately after rinsing and kept at –80 °C. ACE seeds were germinated in new Petri dishes lined with two layers of filter paper wetted with distilled water. The dishes were placed in the dark at 20 °C for 2 weeks. Germinated seeds (about 20%) were removed and those remaining were considered to be dormant. A small number of the dormant seeds were cooled to 4 °C for 48 h to break dormancy and then, after transfer to 20 °C, allowed to complete germination to test for seed viability. The remainder were frozen in liquid nitrogen and stored at –80 °C. The induction of secondary dormancy was repeated three times.

Induction of secondary seed dormancy in *Arabidopsis*

The protocol for induction of secondary dormancy in seeds of wild type *Arabidopsis thaliana* (L.) Heynh Landsberg erecta and mutants (*aba-1-1*, ABA-deficient and *abi1-1*, ABA-insensitive) was almost identical to that described for *B. napus* seeds above, expect for the duration of induction. Based on our preliminary test, the highest percentage of *Arabidopsis* seeds entered dormancy after PEG treatment for 3 weeks (data not shown).

Microarray analysis

Protocols for total RNA extraction, cDNA synthesis and labeling, microarray hybridization and scanning, analyses of microarray data, and confirmation of microarray results by real-time PCR, were described in Fei et al. (2007). Initial selection of genes for further analysis was based on criteria of both statistical significance ($P \leq 0.05$) and biological significance (two-fold difference in expression between samples).

Abscisic acid analysis

The frozen seeds were ground into fine powder in liquid nitrogen and lyophilized for 48 h. Approximately 100 mg dry mass (DM) of each sample (exact weight was recorded) was used for abscisic acid (ABA) analysis. The procedures for sample extraction, purification, and ABA quantification were described in Chiwocha et al. (2003).

Sample preparation for nontargeted metabolite profiling

Frozen PEG-treated DH and ACE seeds (50 mg), each of which consisted of seeds pooled from replicate treatments, were homogenized and then extracted in duplicates with 1 mL of 1% (*v*/*v*) formic acid (aqueous extract) and then with 3 × 3 mL ethyl acetate (organic extract) and stored at –80 °C. The combined ethyl acetate fractions were dried under a stream of nitrogen, dissolved in 1 mL of methanol and stored at –80 °C until Fourier transform mass spectrometry (FTMS) analysis. Samples were diluted 1:19 (*v*/*v*) prior to analysis, using sample ionization by electrospray (ESI) and atmospheric pressure (APCI) methods. Solvent mixtures consisting of 50/50 (*v*/*v*) MeOH – 0.1% ammonium hydroxide or 50/50 MeOH – 0.1% formic acid were used as mobile phases for dilution of all negative and positive ionization analyses, respectively, as described by Aharoni et al. (2002).

Fourier transform – ion cyclotron resonance mass spectrometry

All analyses were performed using a Bruker Daltonics (Billerica, Mass.) APEX III Fourier transform-ion cyclotron

resonance (FT-ICR) mass spectrometer equipped with a 7.0 T actively shielded superconducting magnet. Samples were introduced separately by direct injection into ESI and APCI sources with positive and negative sources at a flow rate of 600 $\mu L \cdot h^{-1}$. The instrument conditions were tuned to optimize ion intensity and broad-band accumulation over the mass range of 100–1000 Da. Using a linear least-squares regression line, mass axis values were calibrated such that each internal standard mass peak had a mass error of < 1 ppm compared with the theoretic mass. Using XMASS software (version 6.0.3) from Bruker Daltonics, the mass spectra from each analysis were integrated to create a peak list with the accurate mass and absolute intensity (signal to noise intensity) of each peak. Compounds in the range of 70–1000 *m/z* were analyzed. All detected masses were converted to corresponding neutral masses (i.e., assuming hydrogen adduct formation) to compare and summarize data across ionization modes and polarities. A self-generated two-dimensional (i.e., mass versus sample intensity) array was created using *DISCOVA*metrics (version 4.0) software (Phenomenome Discoveries, Saskatoon, Sask.). Data from multiple files were integrated and the combined file was processed to identify unique masses. The average, representing the *y*-axis, of each unique mass was determined in a column and a row, representing the *x*-axis, was created for each file selected for analysis. The signal-to-noise intensity for each mass in selected files, representing the analyzed samples was then entered into its representative *x*,*y* coordinate.

Further details of sample preparation and analysis, data processing of samples for nontargeted metabolite analysis can be found in Zulak et al. (2007).

Statistical analysis and putative identifications

Two technical replicates of PEG samples from both DH and ACE, consisting of the independent extraction and analysis of one biological replicate from pooled seeds, were used in Student's *t* test analysis (*DISCOVA*metrics software) to determine whether the means of metabolites in the two cultivars were different. First, detected masses were filtered for ^{13}C isotopes and putative adducts and then filtered mass signal to noise intensities were compared between the two cultivars. Significant (Student's *t* test $P \leq 0.1$) masses were determined and their putative identifications were made by searching public databases such as ChemFinder (www.chem-Wnder.cambridgesoft.com), CHEMnetBASE (www.chemnetbase.com), PubChem Compound (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=pccompound, and Lipid maps (www.lipidmaps.org) with a mass variance (error) of < 3 ppm for target metabolites. The dataset of deduced molecular formula was also used to search a custom list of predicted plant metabolites.

Results and discussion

Overall gene expression trends

Seeds of both cultivars were treated with PEG for 4 weeks. The nondormant DH seeds were collected for RNA extraction immediately after removal of PEG (i.e., before changes in gene expression associated with the completion of germination occurred), whereas the dormant ACE seeds (after removal of PEG) were incubated until the small number of nondormant seeds had germinated and were removed. RNA was extracted from the remaining dormant ACE seeds. Therefore, our experiments compare dormant ACE seeds with nondormant DH12075 seeds prior to germination of the latter.

At the time these experiments were performed, *Brassica* microarrays were not available and preliminary experiments indicated that *Arabidopsis* cDNA arrays were more sensitive than oligonucleotide arrays. *Brassica napus* and *Arabidopsis* both belong to the Brassicaceae family and sequence identity in corresponding gene sequences is around 86% (Parkin et al. 2005). Thus, it is possible to study gene expression in Brassicas by using genomic tools developed for *Arabidopsis* as in previous reports (Li et al. 2005; Fei et al. 2007). In this report, *B. napus* genes hybridizing to probe spots are referred to by means of their *Arabidopsis* homologs. For independent confirmation of microarray ratios, 19 genes from microarray data (including up- and down-regulated, and unchanged genes) were analyzed by two-step real-time PCR. The expression ratios of selected genes from real-time PCR were highly correlated with those from microarray data (correlation co-efficient of 0.7; see supplementary data,[3] Fig. S1), confirming the reliability of microarray data.

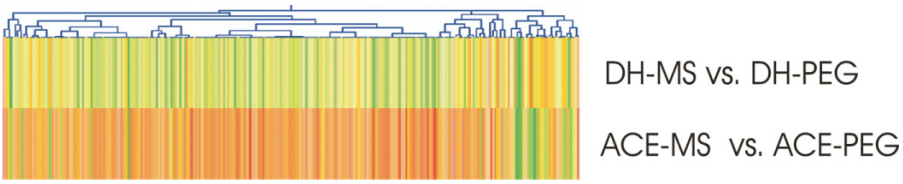
For each cultivar, expression was compared with the corresponding mature stage seed. It was established in a previous study that there were almost no significant differences in gene expression between mature stage seeds of the two cultivars under consideration (Fei et al. 2007). We also performed direct comparisons between PEG-treated seeds of both cultivars. The results obtained were consistent with results of the comparisons using dry seed as a reference stage, and so results of the direct comparisons are not presented except in a few specific cases to provide additional correlative support for trends in gene expression.

All gene expression levels from the six replicates were statistically analyzed using Significance Analysis of Microarrays (SAM) software (Tusher et al. 2001). For the genes that were statistically significantly different ($P \leq 0.01$) between cultivars, an additional criterion of biological significance was applied by using a minimum two-fold threshold ratio. Lists of significantly differentially expressed genes were prepared based on both statistical and biological criteria. However, any ratio threshold is arbitrary and in many cases expression ratios below two-fold are functionally significant. Therefore, we selectively supplemented our analysis below with genes that fall short of the biological criterion, but whose expression changes are consistent with those of functionally related genes that meet the two-fold threshold.

Following PEG treatment, there were 157 and 228 genes that were significantly differentially expressed in DH and ACE seeds, respectively. Hierarchical clustering analysis

[3] Supplementary data for this article are available on the journal Web site (http://botany.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 3942. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/eng/ibp/cisti/collection/unpublished-data.html.

Fig. 1. Hierarchical clustering of genes whose expression is significantly altered by treatment with a –1.5 MPa of PEG solution for 4 weeks to induce secondary dormancy.



showed that there were more down-regulated genes in PEG-treated DH seeds (119 of 157 genes), and more up-regulated genes in PEG-treated ACE seeds (186 of 228) (Fig. 1). By comparing the up-regulated genes in DH and ACE seeds, we identified 28 genes that were up-regulated in both cultivars (commonly up-regulated) and 10 and 158 genes that were specifically up-regulated in DH and ACE, respectively. Among down-regulated genes, 23 genes were commonly down-regulated, whereas 96 and 19 genes were specifically down-regulated in DH and ACE seeds, respectively (Fig. 2).

Fig. 2. Venn diagram showing the statistically and biologically significant genes commonly and specifically expressed in PEG treatment of DH (red) and ACE (green) seeds. Yellow indicates genes expressed in both cultivars.

Gene functions and differential expression between cultivars

Among the 28 commonly up-regulated genes, there was a significant number involved in metabolism of carbohydrates and proteins, as well as response to abiotic stimuli and transport (see supplementary data,[3] Table S1). However, among the 23 commonly down-regulated genes, few were associated with metabolism and response to stress; many were not associated with known biological processes (see supplementary data,[3] Table S2).

Only 10 genes were specifically up-regulated in DH seeds (see supplementary data,[3] Table S3) of which, one (hybridizing to a probe for At2g40170) codes for a homolog of the late embryogenesis-associated (LEA) gene *AtEM6*. This gene is required for normal seed development (Manfre et al. 2006) and is well known as responsive to ABA (Vicient et al. 2000). In one study, its expression showed no correlation with seed dormancy in *Arabidopsis* (Baumbusch et al. 2004), but in a more recent analysis of *Arabidopsis* transcription, *EM6* was one of a group of LEAs that are expressed at higher levels in both primary and secondary dormant seeds relative to nondormant (afteripened) seeds (Cadman et al. 2006).

Relative to the number of up-regulated genes, there were more genes specifically down-regulated in DH seeds (see supplementary data,[3] Table S4). Among these genes, 37 were mainly associated with metabolism and transport and 15 genes were related to protein biosynthesis and metabolism, suggesting that numerous physiological and biochemical activities were slowed during PEG treatment. The expression of homologs to two transcription factors, *WEREWOLF1* (At5g14750) encoding the MYB family transcription protein *AtMYB66* and *ABF3* (At4g34000) encoding an ABA-response element binding factor protein, were repressed. *WEREWOLF1* is known to be involved in root and hypocotyl epidermal cell fate determination and is expressed at higher levels in nongerminating than in germinating seeds (Li et al. 2005). *ABF3* is one of 75 distinct members of the bZIP family in the *Arabidopsis* genome (Jakoby et al. 2002); it is inducible by ABA and various stress treatments (Choi et al. 2000) and functions in ABA signaling (Kang et al. 2002).

Among the 158 genes specifically up-regulated in ACE (see supplementary data,[3] Table S5), 31 were involved in primary and secondary metabolism, 26 were associated with protein biosynthesis and metabolism, 17 were related to transport, and 6 were related to transcription, indicating active physiological and biochemical processes during induction of secondary dormancy. Six genes in this list were associated with photosynthesis.

Table 1. Genes significantly down-regulated in DH seeds and up-regulated in ACE seeds treated with PEG.

AGI number	Fold change DH	Fold change ACE	Description (TAIR)	Biological process (Nick's classification)
At5g65220	0.43	5.04	Putative 50S ribosomal protein L29	Protein biosynthesis, cell organization and biogenesis
At5g41700	0.29	2.18	Putative E2 ubiquitin-conjugating enzyme UBC8	Protein metabolism
At3g08580	0.32	3.16	Mitochondrial ADP/ATP carrier protein 1	Transport, biological process
At1g11260	0.41	4.94	Glucose transporter (STP1)	Transport
At2g38540	0.44	3.14	Non-specific lipid transfer protein 1	Transport
At2g38530	0.39	5.22	Non-specific lipid transfer protein 2	Transport

Note: AGI, Arabidopsis Genome Initiative database.

Genes that may be directly associated with secondary dormancy

In terms of identifying genes that may be specifically associated with secondary dormancy, it is of particular interest that there were six (At1g11260, At2g38530, At2g38540, At3g08580, At5g41700, At5g65220) highly up-regulated in ACE seeds and down-regulated in DH seeds after PEG treatment (Table 1).

Genes At2g38530 and At2g38540 encode nonspecific lipid transfer protein2 and 1 (nsLTPs), respectively. The nsLTPs exhibit a broad range of substrate specificity capable of transferring several classes of phospholipids, glycolipids, fatty acids and steroids between membranes, with wide-ranging binding affinities (Helmkamp 1986; Wirtz 1991; Kader 1996). The nsLTPs are involved in a variety of biological processes, such as cutin formation, embryogenesis, defense reactions against phytopathogens, symbiosis (Sterk et al. 1991; Molina et al. 1993; Segura et al. 1993; Pyee et al. 1994) and plant adaptation to various environmental conditions such as cold- and drought-stress induction in barley (Dunn et al. 1991), salt-induced expression in tomato (Torres-Schumann et al. 1992), and drought-stress induction in sunflower leaves (Ouvrard et al. 1996). Both *nsLTP2* and *nsLTP1* are ABA-inducible (Garcia-Garrido et al. 1998; Huang et al. 2007). In the latter paper, *nsLTP1* and *2* were shown to be inducible in green tissue by the ABA analog PBI425 (Huang et al. 2007), which hyperinduces ABA responses and therefore reveals ABA-regulated genes more effectively than ABA itself. The different responses of these genes to induction of secondary dormancy in DH and ACE seeds suggest that their expression reflects increased ABA responses and also suggests that metabolic processes involving lipid transport occur at higher levels in ACE than in DH. Differences in lipid metabolism between the cultivars are discussed below.

Gene At5g65220 codes for a putative 50S ribosomal protein L29, which is a component of the large ribosomal subunit and is involved in protein biosynthesis and ribosome biogenesis (Klein et al. 2004). The expression level of this gene matched well with the expression of genes related to protein biosynthesis in DH (see supplementary data,[3] Table S3) and ACE seeds (see supplementary data,[3] Table S5). This gene has recently been shown to be inducible by ABA using the hyperactive ABA analog PBI425 (Huang et al. 2007).

Gene At5g41700 codes for putative E2 ubiquitin-conjugating enzyme (UBC8). Covalent attachment of ubiquitin to other cellular proteins has been implicated in a multitude of diverse physiological processes in eukaryotes including selective protein degradation. This attachment is carried out by a multi-enzyme pathway consisting of three classes of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin-protein ligases (E3s) (Sullivan et al. 1994). In a study of genome-wide ABA-responsive gene expression, 25 genes coding for proteins putatively involved in regulated proteolysis were up-regulated in ABA treated *Arabidopsis*, and 3 of them were putative UBC genes (Hoth et al. 2002). UBC8 has recently been shown to be ABA-inducible in green tissue (Huang et al. 2007). It was suggested that ABA might trigger the controlled degradation of a variety of cellular regulatory proteins via the ubiquitin pathway (Hoth et al. 2002). Our data also showed that there were 18 genes involved in protein metabolism in PEG-treated ACE seeds (see supplementary data,[3] Table S5) in which there was a high level of ABA (Fig. 3).

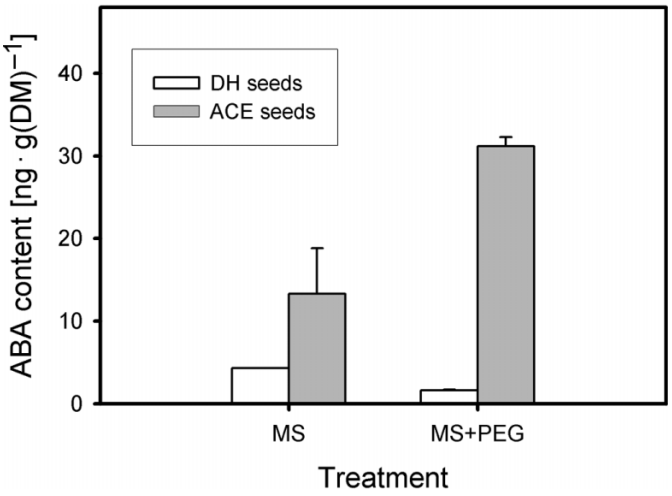
Gene At3g08580 codes for a mitochondrial ADP/ATP carrier protein 1. The ADP/ATP carrier protein is the most abundant protein in mitochondria from different sources (Smagula and Douglas 1988). Mitochondrial adenylate carrier protein serves to supply the cytosol with ATP synthesized at the luminal site of the mitochondria through oxidative phosphorylation. The high expression of this gene in PEG-induced dormant ACE seeds implies an active energy pathway in mitochondria during induction of secondary dormancy.

Gene At1g11260 encodes a proton/hexose cotransporter (STP1) and is therefore an energy dependent sugar transporter (Sauer et al. 1990). The high expression of ADP/ATP carrier protein gene (At3g08580) may be associated with the activity of STP1. It has been reported that high concentrations of glucose inhibit germination of *Arabidopsis* seeds. This inhibition acts on embryo growth and independent of hexokinase function (Dekkers et al. 2004), and is dependent on ABA synthesis (Huijser et al. 2000; Ullah et al. 2002). Evidence supporting this also comes from measurements of ABA in glucose-treated seedlings (Arenas-Huertero et al. 2000) and the observation that ABA synthesis and response mutants are insensitive to glucose (Laby et al. 2000; Rook et al. 2001). Increased expression of *STP1* is consistent with the involvement of ABA and sugar in producing dormancy in ACE.

Abscisic acid and secondary dormancy

It is well established that ABA has a crucial role in promoting and maintaining primary dormancy, but its involve-

Fig. 3. Endogenous ABA levels in mature seeds (MS) of 'DH12075' (DH) and 'AC Excel' (ACE) and in MS seeds treated with –1.5 MPa of polyethylene glycol (PEG). Each value is a mean of three replicates. Bars indicate SE.



ment in secondary dormancy has not been studied to the same extent in *B. napus* (Gulden et al. 2004*a*), although it has recently been shown to be a key factor in both primary and secondary dormancy in *Arabidopsis* (Cadman et al. 2006).

Mature ACE seeds contained almost three-fold more ABA than DH seeds. After 4 weeks of PEG treatment, the level of ABA in ACE seeds further increased about three-fold, (relative to ACE mature seed) but decreased in DH seeds (relative to DH mature seeds) (Fig. 3).

We have already noted a number of ABA-related genes that are expressed at higher levels in dormant ACE seeds than in DH seeds such as *nsLTPs1* and *2*, *UBC8*, *L29*, and *ABF3*. Comparing genes identified from PEG-treated DH and ACE seeds with a comprehensive list of genes regulated by ABA in *A. thaliana* (Huang et al. 2007), we found that many were commonly regulated by ABA and PEG treatments (Fig. 4 and see supplementary data,[3] Tables S6 and S9). Consistent with the difference of ABA concentration in DH and ACE seeds, many more ABA-inducible genes were up-regulated by PEG in ACE seeds or were down regulated by PEG in DH seeds. The ABA-regulated genes that were specific to ACE are shown in Fig. 4. These results suggest that PEG-induced dormancy may be realized through elevated ABA, which in turn regulates the expression of ABA-responsive genes. In addition to those genes noted above, other ABA-related genes that were specifically up-regulated in PEG-treated ACE include *PHOPHOLIPASE D* delta (At4g35790) and *LATE EMBRYOGENESIS ABUNDANT3* (*LEA3*, At4g02380). However, it must be noted that many genes up-regulated specifically in ACE are not related to ABA and indeed some are down-regulated by most of the ABA-related treatments in Huang et al. (2007). Consequently, changes that were specific to ACE were not all related to ABA. Finally, genes commonly up-regulated in both cultivars were not ABA-regulated (see supplementary data,[3] Table S9); therefore, the set of ABA-regulated genes is specific to PEG-treated ACE.

PEG treatment in ACE only affects a subset of ABA responsive genes – some are unaffected or down regulated. We previously noted *EM6*, which is expressed more in DH, as well as *RAB18*, which is down regulated in both DH and ACE by PEG treatment. Other examples include the dehydration-induced gene *ERD15* (At2g41430) that is induced in *Arabidopsis* vegetative tissue by 24 h treatment with ABA or the ABA analog PBI425, but is unchanged in any of the *Brassica* seed comparisons (see supplementary data,[3] Table S9). Similarly, the benzodiazepine receptor-related gene (At2g47770) is strongly induced by all ABA treatments but is unaffected by all *Brassica* seed comparisons, although there is a weak specific down-regulation in PEG-treated ACE seeds (Huang et al. 2007).

It had previously been established that both high temperature treatment and moist chilling in the dark induced secondary dormancy in *Arabidopsis* (Toorop et al. 2005; Cadman et al. 2006). To provide genetic evidence for a regulatory role of ABA in secondary dormancy, we first established that the PEG treatment used for induction of secondary dormancy in *B. napus* also worked in *Arabidopsis* seeds. Our results documented a high rate of induction of secondary dormancy by PEG in wild type *Arabidopsis* seeds. However, induction of secondary dormancy in the ABA-deficient mutant *aba1-1* (Koornneef et al. 1982; Karssen et al. 1983) was less than 20%. In the ABA-insensitive mutant *abi1-1* (Gilmour and Thomashow 1991), about 60% of seeds were induced into dormancy (Fig. 4). It therefore appears that the induction of secondary dormancy is almost completely dependent on normal levels of ABA biosynthesis and partially dependent on ABA signaling via the ABI1 protein phosphatase. It may be concluded that ABA plays a key role in PEG-inducted secondary dormancy.

It has been established in *Arabidopsis* that ABA does not inhibit storage reserve mobilization in nongerminating seeds (Pritchard et al. 2002). Assuming that this is also true in *B. napus*, the activity of ABA in no way prevents storage reserve hydrolysis and energy metabolism in dormant seeds. Therefore, there is no inconsistency between higher transcriptional and metabolic activity in ACE and more pronounced ABA-induced effects.

Associating differences in gene expression with differences in metabolite contents

The data from nontargeted metabolite analyses allowed us to verify the significance of some of the differences in gene expression by mapping genes and metabolite data onto pathways. Nontargeted metabolite analysis using four different modes of analysis [positive and negative electrospray ionization (ESI) and atmospheric pressure ionization (APCI)] from both aqueous and organic extracts detected 433 analytes in both cultivars following PEG treatment. Among them ~88 analytes were significantly different (Student's t test $P < 0.1$) between the two cultivars, 72 of which accumulated preferentially in ACE. The relative intensities (ratio of DH / ACE) varied from 3.2 to 0.18. After removal of masses that could not be assigned a putative identity, the remaining ones that were significantly different are listed in the supplementary data,[3] Table S7. Of particular interest are lipids, phenylpropanoids, and sugars that are enriched more in ACE than in DH.

Four phenylpropanoid-containing moieties with detected

Fig. 4. Abscisic acid (ABE) inducibility of genes up-regulated specifically in 'AC Excel' (ACE). Plot of gene expression vs. treatments for a cluster of ABA-regulated genes expressed specifically in ACE. ABA data is from Huang et al. (2007). *Brassica* seed data are on the left panel. Treatments of *Arabidopsis* seedlings with ABA (6 h and 24 h) and PBI425 (6 h and 24 h) are in the right panel. Genes included in this cluster are listed in the supplementary data,[3] Table S6.

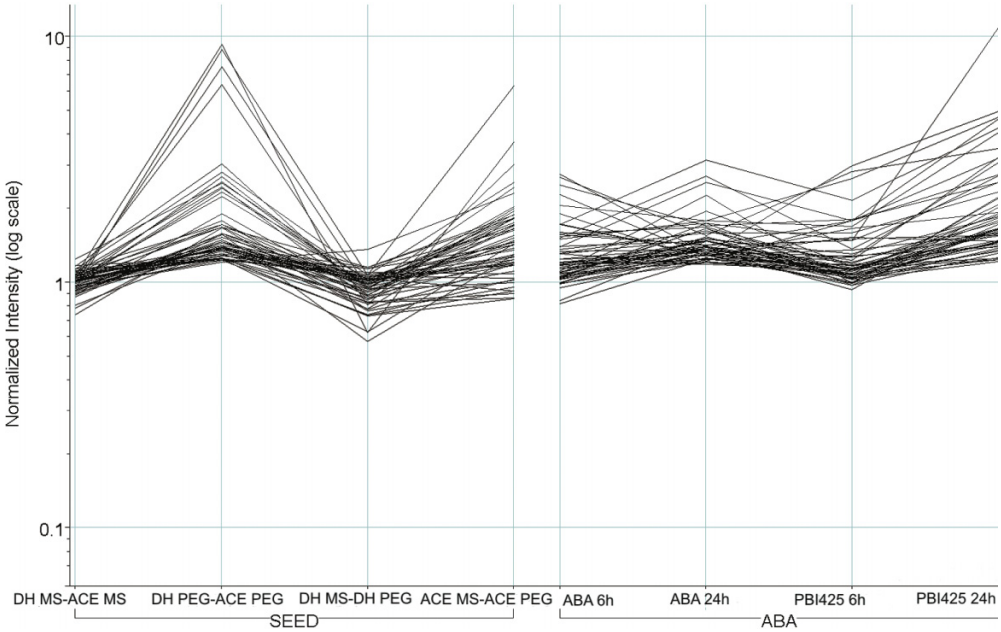
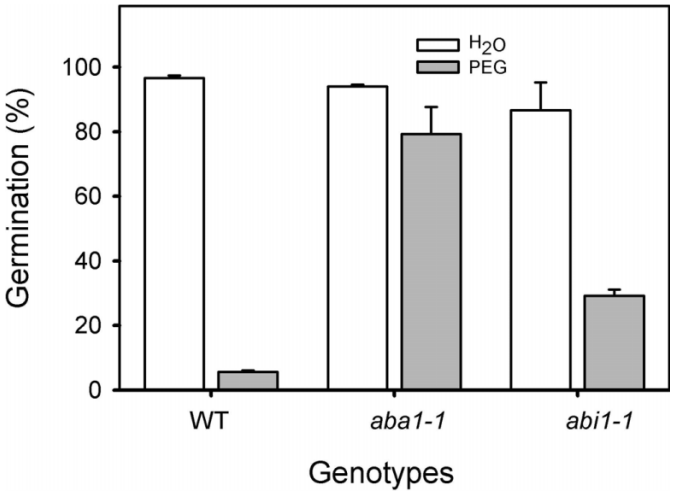


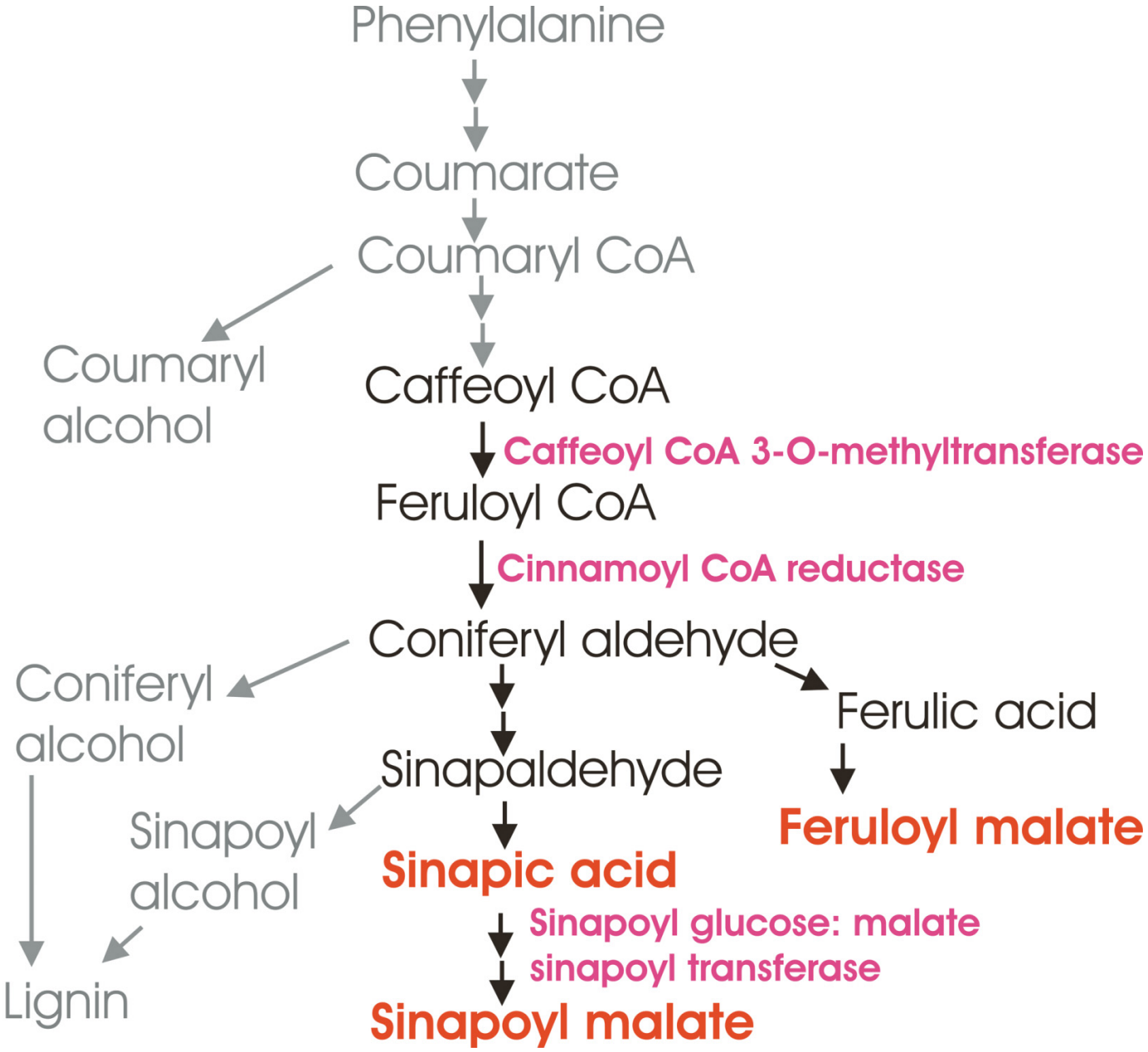
Fig. 5. Germination of *Arabidopsis* seeds (wild type and mutant genotypes) treated with –1.5 MPa of polyethylene glycol (PEG) solution in the dark at 20 °C for 3 weeks and then transferred to water for 2 weeks. Germination in water was used as control for each genotype. Each value is a mean of three replicates. Each replicate contained a minimum of 100 seeds. Bars indicate SE.



masses of 978.2612, 224.0684, 340.0802, and 310.0695 accumulated preferentially in ACE. Among these are sinapic acid (mass = 224.0685), as well as the malic acid conjugate of sinapic acid (340.0794). Two other phenylpropanoids accumulated, one whose identity is ambiguous (mass = 978.2612) and the other is a malate conjugate of 4-hydroxy, 3-methoxy cinnamic acid (feruloyl malate). The presence of the four phenylpropanoids suggests more phenylpropanoid metabolism in ACE. Among corresponding genes, the difference in expression of caffeoyl CoA 3-O-methyltransferase (At4g34050) between cultivars is likely significant. This gene codes for an enzyme that converts caffeoyl CoA to feruloyl CoA, which is then converted to coniferyl aldehyde by cinammoyl CoA reductase, which is also up-regulated specifically in ACE, although it does not reach the two-fold criterion for biological significance (see supplementary data,[3] Table S8). Coniferyl aldehyde is converted to various products including sinapic acid. Conjugation of sinapate to form sinapoyl malate via the sinapate ester pathway is likely related to increased expression of sinapoylglucose:malate sinapoyltransferase (*SNG1*) (At2g22990) (supplementary data,[3] Table S8). Esters of hydroxycinnamic acids (especially sinapate) are major soluble phenypropanoid metabolites in Brassicaceae and serve as antinutritionals in seed (Naczk et al. 1998). These results are summarized in Fig. 6 and indicate increased activity of the pathway from caffeoyl CoA to malate esters of hydroxycinnamic acids in ACE. Increased expression of a homolog of isoflavone reductase (At1g19540) in ACE (supplementary data,[3] Table S8) also suggests higher phenylpropanoid metabolism.

With respect to lipids, there were three lipid-transfer genes more highly expressed in ACE (see supplementary data,[3] Table S5). As noted above, LTPs are potentially involved in many processes and their presence is consistent with increased lipid levels and therefore an increased requirement for lipid relocation within cells. Furthermore, higher expression of both oleosin 1 and 2 suggests more lipid bodies are required for oil (triacylglycerol; TAG) storage. There were also higher levels of two desaturases At3g12120 (*FAD2*; *FATTY ACID DESATURASE2*, responsible for 18:2 fatty acid synthesis) and At3g61580 (coding for a sphingolipid desaturase), suggesting increased fatty acid modification, which is consistent with generally higher lipid synthesis. A third desaturase *FAD3* (*OMEGA-3 FATTY*

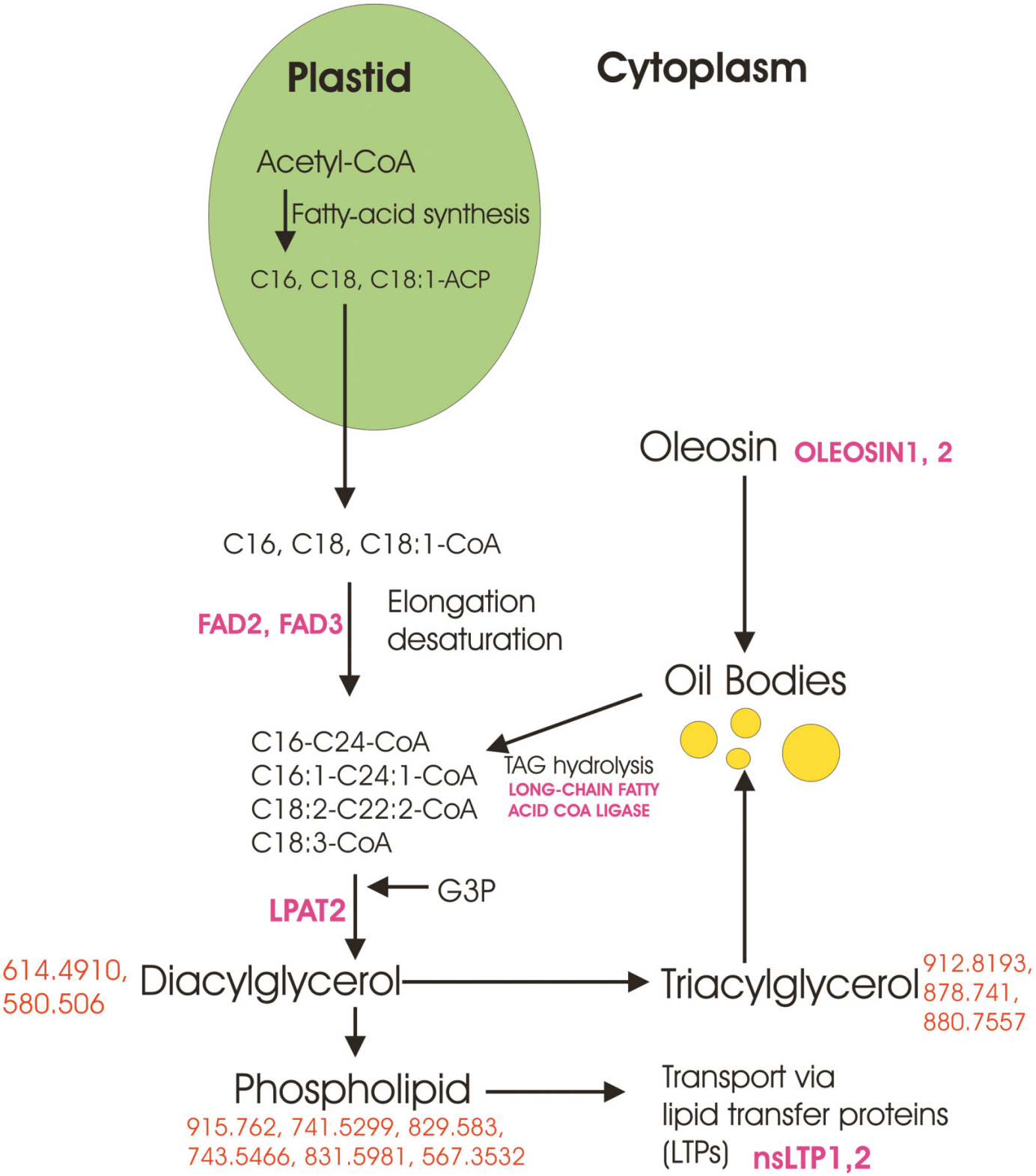
Fig. 6. Summary of metabolites and genes of the phenylpropanoid pathway that are at higher levels in 'AC Excel'. Enzyme activities coded by genes with higher expression in 'AC Excel' are shown in bold magenta and metabolites in red. The genes and metabolites together indicate more conversion of caffeoyl CoA into sinapoyl and feruloyl malate.



ACID DESATURASE, At2g29980) also increased in ACE (see supplementary data, Table S8). Also consistent with increased lipid formation in ACE was higher expression of *LONG-CHAIN FATTY ACID COA LIGASE* (At4g23850) (see supplementary data,[3] Table S8).

We noted earlier that *nsLTPs 1* and *2* are ABA inducible, and it has been shown that oleosin gene expression is also induced by ABA in *B. napus* (Zou et al. 1995). Among the metabolites, there were two diacylglycerols (masses 614.4910, 580.5061) which are precursors of phospholipids such as phosphatidyl choline (PC, N, *N*-dimethyl diacylglycerophosphoethanolamines) and phosphatidyl ethanolamine (PE, diacylglycerophosphoethanolamines), as well as TAGs [but not of phosphatidyl serine (PS) or phosphatidyl inositol (PI)]. There are three TAG species (masses 912.8193; 878.741; 880.7557) that accumulated preferentially in ACE, as well as six PE or PC species (masses 915.762; 741.5299; 829.583; 743.5466; 831.5981; 567.3532). A phosphatidyl serine synthase gene (At1g15110) was expressed more strongly in DH than ACE (listed among commonly up-regulated genes in the supplementary data,[3] Table S1). Since phosphatidic acid can be converted either to PS and PI (by PS synthase) or to PE and PC, lower expression of PS synthase in ACE is consistent with more production of PEs in ACE. Among genes that do not reach the two-fold threshold for biological significance, a homolog of a putative *LPAT2*

Fig. 7. Summary of metabolites and genes associated with lipid metabolism that are higher in 'AC Excel'. Genes are shown in bold, capitalized magenta, and the masses of respective lipids detected are in red.



(*ACYL-CoA:1-ACYLGLYCEROL-3-PHOSPHATE ACYLTRANSFERASE2*; At3g57650) was significantly higher expressed in ACE than DH. So overall, there is evidence for more phospholipid metabolism resulting in accumulation of phospholipids (especially PE and PC-types) and TAG accumulation in ACE. These results are summarized in Fig. 7.

There is clear evidence for altered sugar and oligosaccharide metabolism in ACE with the accumulation of di-, tri-

and tetra-saccharides (masses 342.1158; 504.1682; 666.2218) and the precursor metabolite UDP-glucose, whereas a sugar acid (mass 196.0584) accumulated preferentially in DH. Correspondingly, several sugar-related genes were expressed more strongly in ACE than DH, including the glucose transporter *STP1* (noted above) and *CELLULOSE SYNTHASE* (At5g09870), as well as two glycosyl hydrolases (At1g02640, At5g49360) and a chitinase (At2g43590). In addition, there are several sugar-related genes that were significantly induced in ACE but do not meet the two-fold criterion of biological significance (see supplementary data,[3] Table S8). These include three more glycosyl hydrolases (At5g26000, At3g07160, At5g11920), a glucanase (At1g64390), a *XYLOGLUCAN:XYLOGLUCOSYLTRANSFERASE* (At5g57560), a *UDP-GALACTOSE// UDP-GLUCOSE TRANSPORTER* (At1g14360), and a *HEXOKINASE* (At2g19860). These changes strongly suggest a greater level of cell wall metabolism (synthesis and degradation) in ACE, and that this results in increased sugar movement and metabolism with an associated accumulation of oligosaccharides. Finch-Savage and Leubner-Metzger (Finch-Savage and Leubner-Metzger 2006) proposed that "germination control by the seed covering layers is achieved through the combined or successive actions of several cell-wall modifying proteins" and there is evidence for the role of β-1,3-glucanases in enabling testa and endosperm rupture. Based on this, it is possible that higher glucan metabolism is related to ACE testa and cell-wall strength.

Conclusions

Although we find numerous differences between the two cultivars, we cannot associate specific differences with dormancy, since the cultivars doubtless differ in other ways that are not phenotypically obvious and there are slight differences in the PEG treatment of the two cultivars. However, among the differences that we report, some are likely to be associated with the potential for secondary dormancy in ACE. Further correlative and functional studies will be required to identify specific metabolic or genetic functions that are causally linked to secondary dormancy.

The differences in responses to PEG between the *B. napus* cultivars can be summarized as follows. In the DH seeds, relatively more genes (119) were down-regulated than up-regulated (38), and many of the down-regulated genes have functions in metabolism, transport, and protein synthesis. In ACE seeds, more genes were up-regulated (186) than down-regulated (42). Among the up-regulated genes, many were associated with active physiological and biochemical processes. Taken together, metabolite and gene expression data suggest that there are significant differences in aspects of lipid, phenylpropanoid, and sugar metabolism between ACE and DH seeds. With respect to lipids, higher levels of PEs and TAGs were detected in ACE and this was associated with increased expression of LTPs, oleosins and fatty acid desaturases. The data on genes and metabolites associated with sugar and phenylpropanoid metabolism suggests more cell wall and secondary product formation in ACE.

It is noteworthy that both dormant and nondormant seeds are transcriptionally, translationally, and metabolically active and are in no sense in a resting or inactive phase. A similar conclusion was reached by Cadman et al. (2006) concerning dormant *Arabidopsis* seeds. Furthermore, analysis of the numbers and functions of differentially expressed genes suggests the induction of different metabolic processes in ACE relative to DH, which are manifested in the detected metabolites. This leads to the counter-intuitive conclusion that seeds require significant changes in gene expression and metabolism to achieve the dormant state. However, in *Arabidopsis* seeds Cadman et al. (2006) found that more genes were up-regulated in after-ripened (nondormant, pre-germination) seeds than in various dormant seeds and that many genes associated with protein synthesis, reserve mobilization and cell wall modification were expressed at lower levels in dormant (primary and secondary) seeds relative to after-ripened seeds. This apparent contrast between *Arabidopsis* and *B. napus* may reflect the different experimental protocols; for example, nondormant seeds in Cadman et al. (2006) were after-ripened for 120 d before imbibition and sampling. Nonetheless, Cadman et al. (2006) documented a role for ABA in maintaining the dormant state and increased expression of stress and ABA-related genes.

ABA levels were higher in mature ACE seeds and PEG-induced ACE dormant seeds than in DH seeds. A subset of ABA-responsive genes was also expressed at higher levels in ACE (Fig. 5) and suggests that ABA is involved in induction of secondary dormancy. ABA may also be a causative factor in the relatively higher amounts of lipid metabolites and genes in ACE. Results from induction of secondary dormancy in *Arabidopsis* WT and ABA mutant seeds further support the idea of ABA involvement. Taken together, PEG-induced seed secondary dormancy appears likely to be realized through an ABA-related mechanism. This process is an active one, requiring increased gene expression and changes in primary and secondary metabolism. This study therefore provides a base for future studies on the mechanistic basis for secondary dormancy.

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