12-Oxo-Phytodienoic Acid Accumulation during Seed Development Represses Seed Germination in Arabidopsis

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Arabidopsis thaliana COMATOSE (CTS) encodes an ABC transporter involved in peroxisomal import of substrates for β-oxidation. Various cts alleles and mutants disrupted in steps of peroxisomal β-oxidation have previously been reported to exhibit a severe block on seed germination. Oxylipin analysis on cts, acyl CoA oxidase1 acyl CoA oxidase2 (acx1 acx2), and keto acyl thiolase2 dry seeds revealed that they contain elevated levels of 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), and JA-Ile. Oxylipin and transcriptomic analysis showed that accumulation of these oxylipins occurs during late seed maturation in cts. Analysis of double mutants generated by crossing cts with mutants in the JA biosynthesis pathway indicate that OPDA, rather than JA or JA-Ile, contributes to the block on germination in cts seeds. We found that OPDA was more effective at inhibiting wild-type germination than was JA and that this effect was independent of CORONATINE INSENSITIVE1 but was synergistic with abscisic acid (ABA). Consistent with this, OPDA treatment increased ABA INSENSITIVES protein abundance in a manner that parallels the inhibitory effect of OPDA and OPDA+ABA on seed germination. These results demonstrate that OPDA acts along with ABA to regulate seed germination in Arabidopsis.

INTRODUCTION

Peroxisomal β-oxidation has multiple roles in plant cells, including catabolism of straight-chain fatty acids derived from storage and membrane lipids, catabolism of branched-chain amino acids, and synthesis of indole-3-acetic acid and jasmonic acid (JA) (reviewed in Baker et al., 2006; Graham, 2008). The Arabidopsis thaliana ATP binding cassette (ABC) transporter COMATOSE (CTS), also known as PXA1 and PED3, is involved in transporting substrates for β-oxidation into the peroxisome (Zolman et al., 2001; Footitt et al., 2002; Hayashi et al., 2002). Peroxisomal enzymes that catalyze the core set of β-oxidation reactions are acyl-CoA oxidase (ACX), multifunctional protein (MFP), and L-3-ketoacyl-CoA thiolase (KAT) (reviewed in Graham, 2008), acx1 acx2, and kat2 mutant seeds are defective in seedling establishment as they are unable to catabolize fatty acids derived from storage oil and require exogenous sucrose as an alternate carbon source to fuel growth until the mutant seedlings become photoautotrophic (Hayashi et al., 1998, 2002; Germain et al., 2001; Zolman et al., 2001; Footitt et al., 2002; Adham et al., 2005; Pinfield-Wells et al., 2005). In addition, these same mutants are severely compromised in germination potential, and this phenotype cannot be rescued by exogenous sucrose (Russell et al., 2000; Footitt et al., 2002, 2006; Pinfield-Wells et al., 2005). Thus, it is not a lack of carbon that is compromising seed germination. The mechanism through which CTS exerts its effect on germination potential has remained unknown since the isolation of the forever dormant cts-1 mutant some 10 years ago in a genetic screen designed to identify mutants with increased seed dormancy (Russell et al., 2000). The nongerminating cts phenotype is not rescued by the germination promoting hormone gibberellic acid (GA) (Russell et al., 2000). Consistent with this observation, genetic studies have shown that cts is epistatic to mutations in RGL2, which encodes a germination-repressing DELLa protein that is targeted for degradation in response to GA (Carrera et al., 2007). Evidence for a direct interaction with the germination-inhibiting phytomone abscisic acid (ABA) recently has been provided through the demonstration that a mutation at the ABA INSENSITIVE5 (ABIS) locus can rescue the impaired germination phenotype of ped3, an allele of cts (Kanai et al., 2010). However, the mechanism by which CTS regulates ABIS transcript abundance in germinating seeds remains unknown. The strong dormant phenotype suggests CTS as a major control point between dormancy and germination, and it has been speculated that CTS facilitates the transport into the peroxisome of an unknown molecule that is required for either the activation or repression of germination (Holdsworth et al., 2008).

The fact that the impaired germination phenotype is also observed in other mutants that are severely compromised in peroxisomal β-oxidation, including kat2, acx1 acx2, and csy2 csy3 (Pinfield-Wells et al., 2005; Pracharoenwattana et al., 2005; Footitt et al., 2006), suggests the actual biochemical process is involved in regulating germination potential. Germination of cts or acx1 acx2 seeds is also not improved by either after-ripening or stratification (Russell et al., 2000; Pinfield-Wells et al., 2005). However nicking of the seed coat or removal of the endosperm or testa does overcome the germination block imposed on the embryo in these mutants, which demonstrates that the embryos...
are viable but in a dormant state (Russell et al., 2000; Pinfield-Wells et al., 2005). Other mutants that are blocked in storage oil breakdown but not in the peroxisomal β-oxidation process itself, including a triacylglycerol lipase mutant sdp1 (Eastmond, 2006) and a peroxisomal long-chain acyl CoA synthetase double mutant, lacs6 lacs7 (Fulda et al., 2004), are not compromised in seed germination but do require exogenous sucrose for normal seedling establishment.

JA is a lipid-derived phytohormone, the synthesis of which is dependent on peroxisomal β-oxidation. As such, it is worthy of investigation as to its possible involvement in the control of germination in mutants disrupted in peroxisomal β-oxidation. JA is involved in regulating various plant biological processes, including stress responses and development. JA, its precursor 12-oxo-phytodienoic acid (OPDA), and associated metabolites, including methyl jasmonate (MeJA) and jasmonoyl-L-isoleucine (JA-Ile) (collectively referred to as jasmonates), are all involved in mediating the stress response to biotic as well as abiotic stimuli (Wasternack and Kombrink, 2010). Besides the stress response, jasmonates are also involved in regulating plant growth and developmental processes, such as root growth, tendril coiling, senescence, glandular trichome development, and reproduction (Staswick et al., 1992; Feys et al., 1994; Xie et al., 1998; Li et al., 2004; Balbi and Devoto, 2008; Wasternack and Kombrink, 2010). The cyclopentenone OPDA possesses signaling properties, some of which are shared with JA and others of which are distinct (Böttcher and Pollmann, 2009). For example, OPDA induces expression of a subset of genes that are not induced by JA (Stintzi et al., 2001; Taki et al., 2005; Mueller et al., 2008; Ribot et al., 2008), and JA-deficient opr3 plants are still capable of exhibiting resistance to insect and fungal pathogen attack, indicating that OPDA in the absence of JA can regulate the defense response (Stintzi et al., 2001). On the other hand, male sterility exhibited by JA-deficient opr3 plants is not reversed by OPDA but only by JA/MeJA (Stintzi et al., 2001). Hence, there are some differences in JA and OPDA effects. The induction of gene expression by OPDA can be via two routes, one that uses part of the JA signaling framework and is CORONATINE INSENSITIVE1 (COI1) dependent and another that is COI1 independent (Stintzi et al., 2001; Taki et al., 2005; Ribot et al., 2008).

JAs belong to a group of compounds called oxylipins that are formed via oxygenation of fatty acids (Acosta and Farmer, 2010). The initial step of JA biosynthesis is the release of the fatty acids α-linolenic acid (18:3) and hexadecatrienoic acid (16:3) from plastidial membrane lipids by lipases, including DEFECTIVE IN ANOTHER DEHISCENCE1 (DAD1) and DONGLE (Ishiguro et al., 2001; Hyun et al., 2008; Ellinger et al., 2010). In the octadecanoid pathway, 18:3 fatty acid is oxygenated in the presence of 13-lipoxygenase (13-LOX) to form 13-hydroperoxylinolenic acid, which is then converted to the cyclic intermediate OPDA by sequential steps catalyzed by allene oxide synthase (AOS) and allene oxide cyclase (AOC) in the plastid. Dinor-oxo-phytodienoic acid (dnOPDA) is a 16-carbon homolog of OPDA that is synthesized from 16:3 fatty acid via a parallel hexadecanoid pathway (Weber et al., 1997; Acosta and Farmer, 2010). OPDA and dnOPDA containing galactolipids have been found in Arabidopsis, indicating that these compounds also exist in an esterified form (Steimach et al., 2001; Hisamatsu et al., 2003, 2005; Andersson et al., 2006; Buseman et al., 2006; Nakajyo et al., 2006; Böttcher and Weiler, 2007; Kourtenko et al., 2007; Glauser et al., 2008). OPDA is transported from the plastid to the peroxisome, where CTS has been shown to be involved in its transport into the peroxisome (Theodoulou et al., 2005). In the peroxisome OPDA is reduced to OPC-8:0 by 12-oxophytodienoate reductase 3 (OPR3) (Sanders et al., 2000; Schaller et al., 2000; Stintzi and Browse, 2000). A 4-coumarate:CoA ligase-like (4CL-like) protein, OPC-8:CoA Ligase1, activates OPC-8:0 to its CoA ester (Koo et al., 2006; Kienow et al., 2008), which then undergoes three rounds of peroxisomal β-oxidation to form JA via the intermediates OPC-6:0 and OPC-4:0. The β-oxidation genes ACX1, ACX5, and KAT2 have been demonstrated to be involved in JA biosynthesis in Arabidopsis (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005; Schilmiller et al., 2007). In Arabidopsis, JAR1 encodes an enzyme that conjugates JA to amino acids (Staswick and Tiryaki, 2004). It has been demonstrated that one of these conjugates, JA-Ile, rather than JA or OPDA, plays the crucial role in the well-documented JA response (Thines et al., 2007), where JA-Ile promotes binding of the F-box protein COI1, resulting in the ubiquitin-dependent degradation of jasmonate ZIM domain (JAZ) proteins that repress transcription of JA-responsive genes (Thines et al., 2007).

The initial aim of this work was to quantify the levels of OPDA, JA, and JA-Ile in dry seeds of a range of mutants that are disrupted in different steps in substrate uptake, activation, and peroxisomal β-oxidation and that exhibit varying degrees of dormancy. This led to the unexpected discovery that all three of these compounds are elevated in mutant seeds that are compromised in germination and, from this, the demonstration that OPDA is a key regulator of germination in Arabidopsis.

RESULTS

**cts, acx1 acx2, and kat2 Seeds Contain Elevated Levels of Oxylipins**

Previous analysis of cts, acx1, and kat2 mutants and transgenic antisense lines have demonstrated that the CTS transporter and peroxisomal β-oxidation are required for wound-induced JA biosynthesis in Arabidopsis leaves (Cruz-Castillo et al., 2004; Pinfield-Wells et al., 2005; Theodoulou et al., 2005). Oxylipin levels were measured in wild-type and mutant seeds to establish if there is any correlation between these signaling molecules and seed germination. Unexpectedly, instead of decreased oxylipins, the dry seeds of cts-2, pxa1-1, acx1-2 acx2-1, and kat2-1 all contained elevated levels of the JA precursor OPDA and JA in comparison with wild-type seeds (Figures 1A and 1B). cts-2 seeds had 3-fold more OPDA and 7-fold more JA compared with the Wassilewskija (Ws) wild type. In addition, JA-Ile levels were elevated to a similar degree in cts-2, pxa1-1, and kat2-1 compared with wild-type seeds (Figure 1C). By contrast, oxylipin levels in seeds of the acx1-2 and acx2-1 single mutants and the lacs6 lacs7 double mutant were the same as, or slightly above, oxylipin levels in the wild-type seeds (Figures 1A to 1C).

Germination frequencies for these various mutants were determined for the same seed batches as used for oxylipin analysis
Figure 1. Oxylipin Analysis and Germination of Various Mutants in the Fatty Acid Catabolism Pathway.

(A) to (C) OPDA levels (A), JA levels (B), and JA-Ile levels (C) in mutant and wild-type freshly harvested dry seeds. (D) Germination frequencies of freshly harvested, stratified seeds. Germination was scored 7 d after placing under germination conditions. (E) to (G) OPDA levels (E), JA levels (F), and JA-Ile levels (G) in 3-month after-ripened Ws and cts-2 seeds. DAI, days after imbibition; DW, dry weight. Mean values of three biological replicates for (A) to (D) and of four replicates for (E) to (G) are shown; error bars represent se. kat2, cts-2, and lacs6 lacs7 are in the Ws background; acx1-2, acx2-1, acx1-2 acx2-1, and pxa1-1 are in the Col-0 background.

(Figure 1D). Consistent with previously published results (Pinfield-Wells et al., 2005; Footitt et al., 2006), kat2, cts-2, acx1-2 acx2-1, and pxa1-1 were all severely compromised in seed germination, whereas the acx1-2 and acx2-1 single mutants germinated at a lower frequency compared with wild-type Ws seeds, but this was not as severe a block on germination as exhibited by the cts-2 and kat2-1 seeds (Figure 1D). Most strikingly, there is a strong inverse correlation between the levels of oxylipins in dry seeds and germination frequency (Figures 1A to 1D). This relationship is remarkably consistent across the wild-type and mutant seed batches shown in Figure 1 with Pearson correlation coefficients of $-0.84$, $-0.76$, and $-0.67$ for germination frequency and levels of OPDA, JA, and JA-Ile, respectively. Thus, it is possible that one or more of these oxylipins is directly involved in the inhibition of germination in the mutant seeds.

We also measured OPDA, JA, and JA-Ile in 3-month-old after-ripened cts-2 and Ws wild-type seeds and found that whereas the levels decrease compared with freshly harvested seed (cf. Figures 1A to 1C with Figures 1E to 1G), the overall pattern of significantly higher levels of oxylipins in cts-2 remains (Figures 1E to 1G). Levels of OPDA and JA decrease upon imbibition of the after-ripened seeds, where by 2 d after imbibition, they fall to about half the levels found in cts-2 dry seeds. Again, the levels of both OPDA and JA are much lower in the Ws wild type than in cts-2 following imbibition (Figures 1E and 1F).

OPDA Rather Than JA or JA-Ile Is Involved in the Inhibition of cts Germination

To determine whether one or more of the oxylipins contributes toward the germination phenotype observed in cts seeds, we crossed the cts-2 and pxa1-1 alleles with mutants in the JA
biosynthesis pathway. The aos mutant (Park et al., 2002), which is disrupted in an early step in the oxylipin pathway prior to OPDA (Figure 2A), was crossed with pxa1-1 in the Columbia (Col) background. As expected, JA, JA-Ile, and OPDA could not be detected in aos, and these compounds were also completely absent from the pxa1-1 aos double mutant despite the fact that their levels are elevated in pxa1-1 (Figures 2B to 2D). Seeds of the aos mutant showed germination at similar frequencies as the wild type, both with and without stratification (Figure 2E). Without stratification, pxa1-1 aos double mutant seeds germinated slightly better than the wild type, whereas stratified pxa1-1 aos seeds germinated similar to the wild type (Figure 2E). Thus, aos is epistatic to pxa1-1 both in terms of oxylipin levels and the inhibition of seed germination phenotype. The opr3-1 mutant (Stintzi and Browse, 2000), which is disrupted in the peroxisomal reduction of OPDA to OPC-8:0 (Figure 2A), was crossed with cts-2 in the Ws background. As expected based on gene function, seeds of opr3-1 were deficient in JA and JA-Ile but contained more OPDA than the wild type (Figures 2B to 2D). The cts-2 opr3-1 double mutant seed contained approximately three times the amount of OPDA in the opr3-1 seed (Figure 2B), and JA and JA-Ile levels were decreased to low levels similar to the Ws wild type (Figures 2C and 2D). Most importantly, germination was not rescued in the cts-2 opr3-1 double mutant, which similar to cts-2, did not show any increase in germination even after stratification (Figure 2E). These results strongly suggest that OPDA rather than JA or JA-Ile is the key player contributing toward the germination phenotype of cts/pxa1 seeds, since OPDA but not JA or JA-Ile accumulates in the nongerminating cts-2 opr3-1 seeds, whereas the OPDA-deficient pxa1-1 aos seeds germinate well. The amount of OPDA in cts-2 opr3-1 seeds was equal to the sum of the amounts of OPDA in seeds of each single mutant separately (Figure 2B), suggesting that the cts-2 and opr3-1 mutations are additive in this respect.

Figure 2. Oxylipin Analysis and Germination Assays of Double Mutants.

(A) JA biosynthesis pathway in Arabidopsis. Dashed arrows indicate route to JA biosynthesis via dnOPDA.

(B) to (D) OPDA levels (B), JA levels (C), and JA-Ile levels (D) in mutant and wild-type dry seeds. Asterisk indicates not detected. Mean values of four biological replicates are shown; error bars represent SE.

(E) Germination frequencies of mutant and wild-type seeds with stratification (white bars) and without stratification (black bars) treatment. Germination was scored 7 d after placing under germination conditions. Five replicates for germination assays are shown; error bars represent SE. Freshly harvested seeds were used for germination assays and oxylipin quantification.
We also measured dnOPDA, the 16-carbon homolog of OPDA that is synthesized from 16:3 fatty acid via a parallel hexadecanoid pathway (Figure 2A; Weber et al., 1997; Acosta and Farmer, 2010), to establish if it showed any correlation with seed germination in the mutants. Quantitative detection of a peak at mass-to-charge ratio (m/z) 265 corresponding to the protonated ion for dnOPDA revealed that the amount in pxa1-1 seeds is similar to Ws, whereas the amount in pxa1-1 is only slightly more than in Col-0 (see Supplemental Figure 1 online). Thus, there is not the same correlation with impaired germination as for OPDA; therefore, we conclude that dnOPDA does not contribute to the impaired germination phenotype.

**OPDA Is a More Potent Inhibitor of Germination Than JA**

Next, we conducted germination assays after incubating Col-0 and Ws wild-type seeds with exogenous JA and OPDA to determine whether these two compounds had any effect on wild-type seed germination. Germination was scored at 2 and 7 d after incubation under germination conditions. We found that OPDA was ~10 times more effective at inhibiting germination than JA was after 2 d incubation (Figures 3A to 3D), with 10 µM OPDA resulting in similar levels of inhibition as 100 µM JA in both Col-0 and Ws and 50 µM OPDA being as effective as 500 µM JA after 2 d of incubation. OPDA (50 µM) was sufficient to inhibit germination completely after 2 d of incubation in Ws. The greater potency of OPDA in inhibiting germination was also observed at 7 d after incubation under germination conditions in Ws, where 500 µM JA was required to cause a similar level of inhibition as elicited by 50 µM OPDA (Figures 3B and 3D). However, in Col-0, the inhibitory effect of OPDA on germination observed at 2 d had been alleviated by 7 d (Figure 3O). We used stratified seeds for all germination assays with OPDA, JA, and ABA to ensure that we were starting with seed batches that were nondormant, and any failure to germinate would be due to the respective treatments. Staswick et al. (1992) and Ellis and Turner (2002) observed that when ABA and JA were both included in the germination medium, they had a synergistic effect on germination inhibition. Hence, we measured germination frequencies when ABA and OPDA were combined in the germination medium at concentrations that had little inhibitory effect separately (Figure 3E). We observed a synergistic effect on germination inhibition when 0.5 µM ABA was combined with either 1 or 2 µM OPDA (Figure 3E), suggesting that these two phytohormones interact to inhibit seed germination.

Penfield et al. (2004) reported that seeds blocked in germination by ABA showed ruptured seed coat and endosperm tissues even though radicle emergence through the micropylar endosperm did not occur. In accordance with that data, we found that 5 µM ABA completely blocks radicle emergence with 76% of the seeds exhibiting ruptured endosperm and seed coats after 7 days in the germination cabinet. However, the viability of seeds that fail to undergo germination/radicle emergence in the presence of OPDA, only 24% of these showed rupture of the seed coat and endosperm tissues with the remainder of non-germinating seeds remaining intact (Figure 3F). We have observed that the non-germinating cts seed also have intact endosperm and seed coats. Thus, the phenotype of non-germinating ABA and OPDA treated seeds is not identical and suggests that endosperm and seed coat rupture plays a more important role in the OPDA block than it does in the ABA block on germination.

**Oxylipins Accumulate in pxa1-1 during Seed Development**

Our data show that cts-2 and pxa1-1 dry seeds contain much higher levels of oxylipins than do the corresponding wild-type seeds (Figures 1A to 1C). We performed oxylipin analysis using developing seeds of Col-0 and pxa1-1 to determine at what point during seed development oxylipin levels accumulate. We found that oxylipin levels in pxa1-1 increased above wild-type levels 15 d after flowering (DAF) (Figure 4). OPDA and JA levels in pxa1-1 were maintained above wild-type levels at 17, 19, and 21 DAF (Figures 4A and 4B), whereas JA-Ile levels in the mutant were higher than wild-type levels at 19 and 21 DAF (Figure 4C). These results indicate that the accumulation of oxylipins in pxa1-1 seeds occurs toward the end of seed development. Oxylipins are also present in developing seeds of Col-0, with OPDA showing a slight increase between 19 and 21 DAF.

**Genes Involved in Oxylipin and GA Biosynthesis Are Induced in Developing Seeds of pxa1-1**

The increase in JA and JA-Ile levels in the peroxisomal ABC transporter and β-oxidation mutants was unexpected since peroxisomal β-oxidation is directly involved in the synthesis of these oxylipins. To investigate further the basis for the increase, we used the Affymetrix ATH1 array to perform a transcriptomic study of Col-0 and pxa1-1 developing seeds at 15 DAF, following which oxylipins begin to accumulate in pxa1-1 (Figure 4). Supplemental Data Set 1 online shows results of rank product (RP) analysis (Gentleman et al., 2004; Hong et al., 2006) of the microarray data set, where genes up- and downregulated in pxa1-1 developing seeds in comparison with Col-0 are ranked with 620 significantly upregulated genes and 591 downregulated genes in pxa1-1 identified after applying a cutoff of 5% false positive prediction parameter. When we classified these genes according to the MIPS MATDB FUCAT functional categories (Ruepp et al., 2004), interestingly for the upregulated genes, among the most highly represented categories are cellular sensing and response to external stimulus, photosynthesis, and plant hormonal regulation (see Supplemental Data Set 2 online). For the downregulated genes, storage protein, stress response, and embryonal development were among the most highly represented categories.

We focused on genes related to the metabolism and signaling of oxylipins, ABA, and GA (Table 1; see Supplemental Data Set 3 online). We found three JA-related genes, DAD1, LIPOXYGENASE2 (LOX2), and AOC4, that are upregulated in pxa1-1 3.5-, 2.2-, and 3.4-fold, respectively, relative to the wild type (Table 1). A second AOC gene, AOC1, and the AOS gene were downregulated in pxa1-1 relative to the wild type (see Supplemental Data Set 3 online). Quantitative RT-PCR analysis showed that at 15 and 17 DAF, DAD1 expression in pxa1-1 was 4- and 10-fold more abundant, respectively, than in Col-0, whereas at 19 and 21 DAF, transcript levels in both pxa1-1 and the wild type were much lower (Figure 5A). On the other hand, expression of the β-oxidation genes ACX1, ACX2, KAT2, and MFP2 was
downregulated in *pxa1-1* (Table 1). Quantitative RT-PCR analysis showed that in *pxa1-1*, transcript levels of *ACX1* (Figure 5B) and *KAT2* (Figure 5C) were lower than those of Col-0 at 15 and 17 DAF and then increased by 19 and 21 DAF to levels that were slightly more than the wild type in the case of *ACX1* and similar to the wild type in the case of *KAT2*. Genes encoding other isoforms of ACX and KAT, such as *ACX5*, *KAT1*, and *KAT5*, appear to be upregulated (see Supplemental Data Set 3 online). These results suggest that the early steps in oxylipin biosynthesis are being induced at the transcript level, but the later steps involving peroxisome metabolism do not show a consistent pattern of regulation.
The transcriptomic study also revealed that a number of genes involved in GA biosynthesis, including KS, KAO, GA20ox1, GA20ox2, GA20ox3, and GA20ox4, are all upregulated in pxa1-1 compared with Col-0, while the GA catabolism genes GA2ox3 and GA2ox6 are downregulated in pxa1-1 (Table 1). Quantitative RT-PCR was used to confirm these observations and to determine transcript levels of GA20ox2 and GA2ox6 from 15 to 21 DAF (Figures 5D and 5E). Consistent with the transcriptomic study, GA20ox2 transcripts were found to be more abundant in 15 DAF pxa1-1 seeds compared with Col-0, and this persisted until 17 DAF but then transcripts fell to the limits of detection at 19 and 21 DAF (Figure 5D). Transcript levels of GA2ox6 were 20- and 4-fold less in pxa1-1 compared with the wild type at 15 and 17 DAF, respectively, but levels then increased above those of the wild type by 21 DAF (Figure 5E). These data suggest that GA metabolism is affected by mutation of the CTS locus and raise questions about the levels of GA in the cts mutants.

For ABA-related genes, the pattern was not as consistent as for GA, with genes related to both biosynthesis and deactivation being downregulated in pxa1-1 relative to Col-0 (see Supplemental Data Set 3 online). For ABA signaling-related genes, ABI2, ABI5, SnRK2.2, and SnRK2.3 were all downregulated in pxa1-1, with ABI8 and SnRK2.6 being upregulated. We also compared the up- and downregulated gene sets in the pxa1-1 versus Col-0 microarray data set with the OPDA-specific response genes identified by Taki et al. (2005) and the suite of TGA transcription factor–regulated genes representing the cyclopentenone oxylipin gene induction profile (Mueller et al., 2008) but did not see any consistent trend with our data. We also compared the ped3 versus wild-type data set from Kanai et al. (2010) with the OPDA-specific response genes (Taki et al., 2005) and TGA transcription factor–regulated genes representing the cyclopentenone oxylipin gene induction profile (Mueller et al., 2008) but did not see any significant overlap. This could be due to the differences in tissues and experimental conditions used by different authors for their microarray analyses.

Gibberellins but Not ABA Increase in cts Dry Seeds

We analyzed GA and ABA levels in dry seeds of cts-2 and pxa1-1 and found that cts-2 seeds contained 2-fold more GA4 and 11-fold more GA1 than Ws (Figure 6A). GA4 and GA1 levels in pxa1-1 were 3- and 5-fold higher, respectively, compared with Col-0 (Figure 6A). Unlike GA, ABA content in cts-2 and pxa1-1 dry seeds was similar to the wild type (Figure 6A). We also measured phytohormone levels over a 2-d time course during which stratified cts-2 and Ws seeds were incubated under germination conditions. Although dry seed ABA levels were similar in both cts-2 and Ws, the decrease in ABA levels upon seed imbibition was slower in cts-2 than in Ws, resulting in higher ABA levels for the first day of the time course before returning to wild-type levels by day 2 (Figure 6B). Similar to dry seeds, elevated levels of GA4 and GA1 were observed in cts-2 compared with Ws following imbibition under germination conditions (Figures 6C and 6D). From 1 to 2 d following imbibition, the levels of the main bioactive GA in Arabidopsis, GA4, increased in Ws seeds during the period when the majority of the seeds germinate (Figures 6C and 6E). Levels of GA4 in cts-2 were above those of Ws until 2 d after imbibition, but this was not sufficient to overcome the severe block on germination in cts-2 (Figures 6C and 6E). These data are consistent with our conclusion that OPDA, possibly through interaction with ABA, blocks seed germination in cts, where the increased endogenous GA in dry and imbibed seeds is not sufficient to alleviate this block.

ABI5 Protein Abundance Is Increased upon OPDA Treatment

Kanai et al. (2010) have recently shown that in ped3, which is allelic to cts-2 and pxa1-1, transcript levels of the ABI5 transcription factor remain elevated relative to the wild type upon seed imbibition. Based on this and other observations, they implicate ABI5 in the mechanism by which dormancy and germination is modified in the ped3/cts-2/pxa1-1 mutant. ABI5 is regulated at both the transcript and protein level by ABA (Lopez-Molina et al., 2001). We measured ABI5 protein levels in 35S:HA-ABI5 transgenic lines in control, ABA, OPDA, and ABA+OPDA treatments 1 d after transfer to germination conditions. Consistent with previous reports (Lopez-Molina et al., 2001) ABI5 levels increased when seeds were placed on 5 μM ABA (Figure 7). In addition, we found that 10 μM OPDA treatment alone results in an increase in ABI5 levels (Figure 7). Whereas 0.5 μM ABA and 2 μM OPDA individually do not cause an increase in levels of ABI5,
when provided together, they do result in an increase (Figure 7). These results are consistent with the germination data in Figure 3E where we found synergism between ABA and OPDA in germination inhibition. They are also consistent with and extend the findings of Kanai et al. (2010) by demonstrating that OPDA is directly involved in regulating the levels of ABI5 protein.

**Effect of OPDA on Arabidopsis Seed Germination Is COI1 Independent**

Various reports have shown that OPDA action can be either COI1 dependent (Ribot et al., 2008) or COI1 independent (Stintzi et al., 2001; Taki et al., 2005). We tested the effect of exogenous OPDA on germination of coi1-16 seeds and found that they respond to OPDA treatment with reduction of germination at 10 and 50 μM concentrations (Figure 8A). We also tested coi1-16 germination in JA-containing medium (Figure 8B) and found that similar to wild-type seeds (Figure 3A), coi1-16 germination could be inhibited by JA, but the concentration of exogenous JA required was considerably more than that of OPDA. Thus, both the OPDA and JA inhibition of germination appears to be COI1 independent.

**OPDA’s Influence on Germination under High Temperature Stress**

To test if OPDA has a natural role in promoting dormancy under unfavorable conditions, we checked germination of aos and opr3-1 at temperatures higher than 20°C. We did not observe a major difference between the mutants and wild type at 27°C (Figure 9A), whereas at 31°C, germination of opr3-1 but not of aos was reduced compared with the wild type (Figure 9B). The observation that germination of opr3-1, which contains more OPDA than the wild type (Figure 2B), is reduced under high temperature stress suggests that the higher levels of OPDA in opr3-1 repress germination under certain stress conditions.

**DISCUSSION**

**OPDA Accumulation Impairs Germination of Seeds Disrupted in the Peroxisomal ABC Transporter and Core β-Oxidation Process**

*Arabidopsis* mutants disrupted in either the peroxisomal ABC transporter or in the peroxisomal β-oxidation process exhibit increased seed dormancy. This phenotype cannot be rescued by exogenous sugars, unlike the seedling establishment phenotype of these same mutants, which is due to a block in storage oil breakdown (Pinfield-Wells et al., 2005). However, other mutants disrupted in storage oil breakdown, including sdp1 (Eastmond, 2006) and lacs6 lacs7 (Fulda et al., 2004), remain unaffected in seed germination but do exhibit the typical compromised seedling establishment phenotype. Thus, seedling establishment requires carbon from fatty acid breakdown but seed germination does not. This study has established the basis of the increased seed dormancy phenotype in mutants that are disrupted in the peroxisomal ABC transporter or core β-oxidation process and in so doing uncovered a role for OPDA in the regulation of *Arabidopsis* seed germination.

Quantitative analysis of OPDA, JA, and JA-Ile in dry seeds of the peroxisomal ABC transporter mutants and β-oxidation mutants exhibiting impaired seed germination (Figures 1A to 1D) revealed a strong correlation between germination frequency and elevated levels of OPDA, JA, and JA-Ile. Thus, one or more of these compounds could be affecting germination in seeds disrupted in the

### Table 1. Gene Expression in *pxa1-1* Developing Seeds at 15 DAF Relative to Wild-Type (Col-0) Expression

| Gene Name                | AGI Code     | Expression in Col-0 | Expression in *pxa1-1* | Average Fold Change |
|--------------------------|--------------|---------------------|------------------------|---------------------|
| JA biosynthesis-related genes upregulated in *pxa1-1* |              |                     |                        |                     |
| DAD1 AT2G44810           | 50.1 ± 50    | 174.6 ± 41.4        | 3.5                    |                     |
| LOX2 AT3G45140           | 54.2 ± 35.8  | 121 ± 15            | 2.2                    |                     |
| AOC4 AT1G13280           | 80.4 ± 54.5  | 273 ± 8.1           | 3.4                    |                     |
| β-Oxidation-related genes downregulated in *pxa1-1* |              |                     |                        |                     |
| ACX1 AT4G16760           | 3155 ± 461   | 1247 ± 98.7         | −2.5                   |                     |
| ACX2 AT5G65110           | 2950 ± 319   | 1401 ± 68.6         | −2.1                   |                     |
| KAT2 AT2G33150           | 9722 ± 76.4  | 4813 ± 482          | −2.0                   |                     |
| MFP2 AT3G06860           | 3271 ± 254   | 1303 ± 174          | −2.5                   |                     |
| GA biosynthesis-related genes upregulated in *pxa1-1* |              |                     |                        |                     |
| KS AT1G79460             | 334.5 ± 53.9 | 775 ± 46.8          | 2.3                    |                     |
| KAO AT1G05160            | 805 ± 214    | 4841 ± 525          | 6.0                    |                     |
| GA2ox1 AT4G25420         | 28.56 ± 28.1 | 141.3 ± 41.4        | 4.9                    |                     |
| GA2ox2 AT5G351810        | 104.9 ± 55.4 | 2123 ± 171          | 20.2                   |                     |
| GA2ox3 AT5G07200         | 502.1 ± 192  | 9521 ± 60.9         | 19.0                   |                     |
| GA2ox4 AT1G60980         | 15.49 ± 12.5 | 61.64 ± 3.68        | 4.0                    |                     |
| GA catabolism-related genes downregulated in *pxa1-1* |              |                     |                        |                     |
| GA2ox3 AT2G334555        | 45.69 ± 18.5 | 8.8 ± 3.8           | −5.2                   |                     |
| GA2ox6 AT1G02400         | 921.8 ± 197  | 70.8 ± 27.6         | −13.0                  |                     |

The table shows upregulated JA biosynthesis-related genes, downregulated β-oxidation-related genes, upregulated GA biosynthesis-related genes, and downregulated GA catabolism-related genes in *pxa1-1*. Mean expression values of three replicates ± SD are shown. AGI, Arabidopsis Genome Initiative.
peroxisomal ABC transporter or core \( \beta \)-oxidation process. To determine which, we crossed the appropriate ecotype allele of the peroxisomal ABC transporter mutant with mutants disrupted in the JA biosynthesis pathway. \( pxa1-1 \) was crossed with \( aos \) (Park et al., 2002), which is mutated in a chloroplastic enzyme that catalyzes an early step in the pathway prior to OPDA production. As expected, seeds of the \( pxa1-1 \) \( aos \) double mutant were deficient in OPDA, JA, and JA-Ile, in contrast with the elevated levels of these oxylipins in \( pxa1-1 \) (Figures 2B to 2D). Consistent with our hypothesis that one or more of these oxylipins impairs seed germination, the germination frequency of the \( pxa1-1 \) \( aos \) double mutant was restored to that of the wild type (Figure 2E). Thus, the \( aos \) mutant is epistatic to \( cts \). \( cts-2 \) was crossed to \( opr3-1 \) (Stintzi and Browse, 2000), mutated in a peroxisomal enzyme that catalyzes the conversion of OPDA to OPC-8:0, which is then activated and \( \beta \)-oxidized to form JA (Figure 2A). In contrast with the \( pxa1-1 \) \( aos \) double mutant, the \( cts-2 \) \( opr3-1 \) seeds still exhibit impaired germination typical of \( cts-2 \) seeds (Figure 2E). The fact that \( cts-2 \) \( opr3-1 \) seeds accumulate OPDA to levels that are similar to \( cts-2 \) seeds, but have reduced JA and JA-Ile (Figures 2B to 2D), points to OPDA as the causative factor impairing germination in seeds that are disrupted in the peroxisomal ABC transporter and by extension the core \( \beta \)-oxidation process. Furthermore, our data suggest that there is a threshold above which OPDA needs to rise before there is an effect on germination since seeds of the \( opr3 \) mutant, which germinate at the same frequency as the \( Ws \) wild type, accumulate more OPDA than \( Ws \), but it is still only half the level present in \( cts-2 \) and one-third the level in the \( cts-2 \) \( opr3-1 \) double mutant (Figure 2B).

It is possible that the levels of OPDA in \( opr3-1 \) are regulated by a JA-stimulated positive feedforward mechanism occurring to a lesser extent in the JA-deficient \( opr3-1 \) mutant. This idea was previously put forward by Stenzel et al. (2003), who showed that unwounded \( opr3 \) leaves contain less free OPDA than would be expected as a consequence of the block in OPR3 activity in this mutant. The presence of low levels of JA and JA-Ile in dry seeds

**Figure 5.** Gene Expression during Seed Development.

\( DAD1 \) (A), \( ACX1 \) (B), \( KAT2 \) (C), \( GA20ox2 \) (D), and \( GA2ox6 \) (E) expression in \( Col-0 \) wild type (black bars) and \( pxa1-1 \) (gray bars) developing seeds. For each gene, expression relative to that for \( Col-0 \) at 15 DAF is plotted (relative expression for \( Col-0 \) 15 DAF is 1). Mean values of three biological replicates are shown; error bars represent SE.
of cts-2 opr3-1 (Figures 2C and 2D) suggests either that the opr3-1 mutant phenotype is leaky in the cts-2 background or another OPR isoenzyme is contributing to the conversion of OPDA to JA. Of the three isoenzymes of 12-oxophytodienoate reductase present in Arabidopsis, biochemical studies have shown that OPR3 is the isoform involved in JA biosynthesis (Schaller et al., 2000), which is consistent with the strong JA null phenotype in opr3 mutants (Stintzi and Browse, 2000). It is possible that opr3-1 is leaky since the T-DNA insertion causing the opr3-1 mutation is in an intron region of the OPR3 gene (Stintzi and Browse, 2000).

It has been reported that JA/MeJA is capable of inhibiting seed germination in Brassica napus, Linum usitatissimum, Solanum lycopersicum, and Arabidopsis (Wilén et al., 1991; Miersch et al.,...
OPDA and ABA Act Synergistically to Inhibit Seed Germination

Our data from exogenous treatment with OPDA and ABA demonstrate that these compounds have a synergistic effect on germination inhibition (Figure 3E). Measurement of OPDA in 3 month after-ripened seeds shows that it persists in cts-2 at ~3 times the level found in the Ws wild type, and upon imbibitions, the levels fall in both but the differential between mutant and wild type remains (Figure 1E). We also observed that the decline in ABA levels in imbibed cts-2 seeds is slower than in wild-type seeds, with ABA levels in cts-2 imbibed seeds being higher than the wild type for the first day following imbibition (Figure 6B). Transcript levels of the ABI5 transcription factor, which is involved in ABA signal transduction, are higher in the ABA-accumulating cyp707a1a2a3 triple mutant, whereas those in ABA-deficient aba2 are lower compared with the wild type, indicating that higher endogenous ABA levels lead to higher ABI5 expression (Okamoto et al., 2010). Kanai et al. (2010) have recently shown that in ped3, which is allelic to cts-2 and pxa1-1, ABI5 transcript levels remain elevated relative to the wild type upon seed imbibition. Transcripts encoding the polygalacturonase inhibiting proteins (PGIPs) also remain elevated in ped3 but not in the ped3 abi5 double mutant, which is rescued in the impaired germination phenotype of ped3. The authors suggest that elevated ABI5 transcript in imbibed ped3 seeds results in

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Figure 8. OPDA and JA Inhibit Germination Independently of COI1.
(A) Germination frequency of coi1-16 on increasing concentrations of OPDA.
(B) Germination frequency of coi1-16 on increasing concentrations of JA.
Mean values of three biological replicates for (A) and (B) are shown; error bars represent se. Seeds were cold stratified for 3 d before placing under germination conditions. 2d, 2 d after placing under germination conditions; 7d, 7 d after placing under germination conditions. For (A), germination frequency values obtained for OPDA treatments at 2 d were normalized against those obtained for 0 µM OPDA at 2 d and 7 d normalized against those for 0 µM OPDA at 7 d. Similar normalization was done for the JA treatments in (B).
ABA’s effect is mediated by a number of transcription factors, including ABI3, ABI4, and ABI5 (Lopez-Molina et al., 2001, 2002; Penfield et al., 2004, 2006; Holdsworth et al., 2008). The differences observed between OPDA- and ABA-treated seeds (Figure 3F) could thus be due to OPDA acting specifically through ABI5 on endosperm rupture compared with ABA acting via a number of transcription factors on multiple processes.

A number of genes related to GA biosynthesis, including GA20ox2, were upregulated in pxa1-1 developing seeds relative to Col-0, while genes related to GA deactivation, such as GA2ox6, were downregulated (Table 1, Figures 5D and 5E). Consistent with this altered gene expression in developing seeds, we found that mature dry seeds of both pxa1-1 and cts-2 contained higher levels of bioactive GAs (Figure 6A). However, these elevated GA levels do not alleviate the severe block on germination in the cts/pxa1 mutants. This is in agreement with the observation that exogenous GA is unable to promote cts germination (Russell et al., 2000). Furthermore, Carrera et al. (2007) report that cts is epistatic to rgl2, which is mutated in a DELLA protein that is a negative regulator of GA response and a repressor of seed germination (Lee et al., 2002). Hence, it appears that OPDA, through interaction with ABA, blocks germination of cts seeds and the higher levels of endogenous GA in dry and imbibed seeds are not sufficient to alleviate this block. OPDA, JA, and JA-Ile are also present in wild-type seeds, albeit at lower levels than in the mutants blocked in seed germination. Given the interaction observed between OPDA and ABA in the regulation of seed germination (Figure 3E) and ABI5 protein abundance (Figure 7), it is reasonable to conclude that the regulation of OPDA levels in developing seeds in response to abiotic and biotic signals will play an important part together with ABA in determining the dormancy state of mature seeds. We found that germination of opr3-1 seeds was reduced compared with wild-type seeds when seeds were incubated at 31°C (Figure 9B), indicating that accumulation of higher levels of OPDA results in increased sensitivity to stress conditions, such as high temperature. This observation is consistent with a role for OPDA in promoting dormancy under unfavorable environmental conditions.

### Accumulation of Oxylipins in cts/pxa1 Seeds

The accumulation of higher levels of JA and JA-Ile in cts-2 and pxa1-1 seeds was unexpected and opposite to that observed after wounding in cts leaves, where JA levels were lower than the wild type (Theodoulou et al., 2005), which suggests that the JA biosynthesis pathway differs between leaves and seeds. We found that genes encoding the enzymes DAD1, LOX2, and AOC4 required for earlier chloroplastic steps in JA biosynthesis are upregulated in developing pxa1-1 seeds compared with the Col-0 wild type (Table 1). The upregulation of some of these early JA biosynthesis genes in developing pxa1-1 seeds suggests that there might be an increased flux of precursors including OPDA through the JA biosynthesis pathway. A large amount of OPDA and 6-dOOPA is found esterified in chloroplast galactolipids in Arabidopsis (Stelmach et al., 2001; Hisamatsu et al., 2003, 2005; Andersson et al., 2006; Buseman et al., 2006; Nakajyo et al., 2007). And others have shown that OPDA levels were increased in the cts-2 seed coat compared with wild type (Table 1). The upregulation of some of these early JA biosynthesis genes in developing pxa1-1 seeds suggests that there might be an increased flux of precursors including OPDA through the JA biosynthesis pathway. A large amount of OPDA and 6-dOOPA is found esterified in chloroplast galactolipids in Arabidopsis (Stelmach et al., 2001; Hisamatsu et al., 2003, 2005; Andersson et al., 2006; Buseman et al., 2006; Nakajyo et al., 2007). And others have shown that OPDA levels were increased in the cts-2 seed coat compared with wild type.

![Germination of opr3 Is Compromised at High Temperature.](image)

Figure 9. Germination of opr3 Is Compromised at High Temperature.

Germination of Col-0, aos, Ws, and opr3-1 at 27°C (A) and 31°C (B). Mean values of three biological replicates for (A) and (B) are shown; error bars represent se. Seeds were cold stratified for 3 d before placing under germination conditions.

The phenotype of nongerminating ABA- and OPDA-treated seeds was not identical (Figure 3F), and it appears that endosperm and seed coat rupture plays a more important role in the OPDA block than it does in the ABA block on germination. ABA’s effect is mediated by a number of transcription factors, specifically in the micropylar endosperm through which the radicle has to emerge for germination to proceed (Penfield et al., 2006). We found that OPDA treatments result in increased ABI5 protein levels in 35S:HA-ABI5 seeds (Figure 7); hence, OPDA is able to regulate levels of ABI5 protein. Furthermore, combining 2 μM OPDA with 0.5 μM ABA results in an increase in ABI5 abundance, whereas separate treatments of 2 μM OPDA or 0.5 μM ABA have no obvious effect (Figure 7). Consistent with these data, we observed synergism between ABA and OPDA in germination inhibition at the same concentrations (Figure 3E). Considering together the results of elevated ABI5 transcripts in ped3 by Kanai et al. (2010), germination data (Figure 3E), and ABI5 protein abundance (Figure 7), it would appear that higher OPDA levels in cts/pxa1 either alone or in combination with ABA result in an increase in ABI5 protein levels, which impacts on seed germination. We anticipate that in the case of ped3 abi5 double mutant, which is rescued in germination (Kanai et al., 2010), OPDA levels remain elevated.

Measurement of ABI5 protein levels was performed on whole seeds (embryos plus endosperms), as the number of isolated endosperms required to do endosperm-only preparations was prohibitive for this analysis. Since ABI5 is known to be expressed specifically in the micropylar endosperm through which the radicle has to emerge for germination to proceed (Penfield et al., 2006), it is tempting to speculate that the micropylar region of the endosperm will play an important role in the response to OPDA. The phenotype of nongerminating ABA- and OPDA-treated seeds was not identical (Figure 3F), and it appears that endosperm and seed coat rupture plays a more important role in the OPDA block than it does in the ABA block on germination.
the esterified form in the galactolipids (Hyun et al., 2008; Ellinger et al., 2010). In such a case, increased levels of DAD1 in cts/pxa1 seeds not only be instrumental in release of more linolenic acid (which could be converted to OPDA) but perhaps could also function in releasing more OPDA itself from the oxylipin-containing galactolipids. Thus, OPDA accumulation in cts mutant may arise as a consequence of one or more indirect effects and may not simply be due to a failure in CTS-mediated transport into the peroxisome. The reason why cts mutants accumulate OPDA in the first place therefore remains an open question.

If in cts/pxa1 seeds the increased amount of OPDA is able to enter the peroxisome via a CTS-independent path, it could then be converted to JA in the peroxisome, resulting in more JA being produced. A similar case involving upregulation of the peroxisomal steps of the biosynthetic pathway can be argued for the β-oxidation mutants, where increased JA would result, provided that other isoforms of ACX and KAT are functional in catabolizing the β-oxidation steps in the respective mutants. In Arabidopsis leaves, the ACX1 isoform plays the major role in the production of JA, with the gene being induced in response to wounding (Cruz Castillo et al., 2004) and the acx1-1 mutant showing an 87% reduction of the wound-induced accumulation of JA seen in the wild type (Pinfield Wells et al., 2005). It is not clear which of the other ACX isoforms is responsible for the small but still significant wound-induced increase in JA in acx1-1. Consistent with the view that multiple isoforms of the core β-oxidation enzymes are involved in JA biosynthesis, quantifiable amounts of basal and wounded leaf JA were present in transgenic antisense ACX1 and KAT2 lines (Cruz Castillo et al., 2004). Furthermore, KAT5 has been shown to be induced by wounding and exogenous JA (Cruz Castillo et al., 2004). Also, we found in our microarray data that whereas ACX1, ACX2, and KAT2 were downregulated in pxa1-1 developing seeds, ACX5, KAT1, and KAT5 were upregulated (see Supplemental Data Set 3 online).

CTS-Independent Route for OPDA Transport into the Peroxisome

The accumulation of JA and JA-Ile in cts seeds indicates that an alternate CTS-independent route/s for import of JA precursors into the peroxisome must exist. JA levels in leaves of the cts mutant are reduced but still quantifiable, suggesting the existence of other nonactive CTS-independent transport mechanisms (Theodoulou et al., 2005). Peroxisomal ion trapping of the OPDA anion based on the lipophilicity of OPDA and the differential pH of the cytosol and peroxisome has been put forward as one such mechanism (Theodoulou et al., 2005).

Kienow et al. (2008) report a 4CL-like protein called OPDA-CoA ligase (At5g63380) capable of converting OPDA to its CoA ester. The authors speculate that an alternate CTS-independent route for OPDA import might involve a transport system that includes or operates along with this CoA ligase (Kienow et al., 2008). When we crossed pxa1-1 with opda-Coa ligase (SALK_003233) and measured JA and JA-Ile, the double mutant seeds contained somewhat lower JA levels (157 ± 8 ng/g) compared with pxa1-1 seeds (245 ± 30 ng/g), whereas JA-Ile levels were similar in the double mutant (599 ± 53 ng/g) and in pxa1-1 (692 ± 66 ng/g). These results suggest that in the absence of CTS activity, a small proportion of JA synthesis involving the OPDA-CoA ligase possibly occurs, but it is unlikely that this is a major alternate route for uptake of OPDA into the peroxisome.

Differential Regulation of JA Biosynthesis in Different Tissues

Weber et al. (1997) showed that the relative levels of JA and OPDA differed according to the species and tissue analyzed. Also, constitutive overexpression of AOC leads to accumulation of JA, OPDA, and MeJA in tomato flowers but not in leaves (Miersch et al., 2004). Both these reports suggest that JA biosynthesis regulation differs according to the tissue. Changes in the transcriptome in response to JA also vary according to the tissue, stimuli, and context as reviewed by Pauwells et al. (2009). Plants of a double mutant in the β-oxidation pathway, acx1 acx5, show reduced pollen viability and an inability to increase JA levels upon mechanical wounding, but they are still capable of accumulating high levels of JA on infection by Alternaria brassicicola, suggesting that JA synthesis might involve different enzymes based on the environmental stimuli and developmental cue (Schilmiller et al., 2007). Consistent with this view, DAD1 has been shown to be essential for Arabidopsis fertility (Ishiguro et al., 2001) but is not essential for wound-induced JA biosynthesis (Hyun et al., 2008; Ellinger et al., 2010). The finding that cts/pxa1 seeds accumulate JA whereas wounded cts leaves are decreased in accumulation of JA (Theodoulou et al., 2005) further substantiates the argument that JA biosynthesis is context dependent and might be regulated differentially according to the plant tissue, external stimuli, or developmental cue.

In conclusion, disruption of the CTS locus affects phytohormone levels during seed development and seed imbibition. We report that high levels of OPDA accumulate in cts/pxa1 seeds, which leads to the inhibition of seed germination. The ABI5 transcription factor, which regulates germination by affecting cell wall pectin degradation (Kanai et al., 2010), appears to be a significant player in this process. Data from this study as well as previous publications indicate that JA, JA-Ile, and OPDA are present in seeds of different species (Miersch et al., 2008; Preston et al., 2009). Further investigations are now needed to establish how oxylipin biosynthesis is regulated in seeds and how OPDA in particular acts as a signaling molecule to influence seed developmental processes, including dormancy and germination.

METHODS

Plant Material

Plants of Arabidopsis thaliana ecotypes Col-0, Ws, and various single and double mutants were grown in a greenhouse supplemented with artificial light to give a photoperiod of 16 h light. kat2 (Germain et al., 2001), aos (Park et al., 2002), and T-DNA insertion line SALK_003233 (At5g63380 encoding OPDA CoA ligase) were obtained from the Nottingham Arabidopsis Stock Centre. pxa1-1 (Zolman et al., 2001), acx1-2 (Adham et al., 2005), acx2-1 (Adham et al., 2005), and acx1-2 acx2-1 (Adham et al., 2005) seeds were kindly donated by Bonnie Bartel, cts-2 (Footitt et al., 2002) seeds were a gift from Alison Baker, lac6 lac7 (Fulda et al., 2004) was from John Browse, opr3-1 (Stintzi and Browse, 2000) was from Frederica Theodoulou, and c01-16 (Ellis and Turner, 2002) was from...
John Turner. We are grateful to Nam-Hai Chua for 35S:HA-ABI5 seeds (Lopez-Molina et al., 2002), pxa1-1, axc1-2, axc2-1, axc1-2 axc2-1, and T-DNA insertion line SALK_003233 were in Col-0, and aos and coi1-16 were in the Col-g background. cts-2, kat2, opr3-1, and lacs6 lacs7 were in the Ws background. Seeds were harvested when plants had stopped flowering and siliques had started to dehisc. Plants were kept watered until seeds were collected. Freshly harvested seeds indicate that experiments were performed within 24 h from harvest. Seeds were size sieved using a sieve with mesh size 250 µm.

Sterile opr3-1, aos, pxa1-1 aos, and cts-2 opr3-1 plants were sprayed with 450 µM MeJA (Sigma-Aldrich) in 0.1% Tween 20 to obtain seed.

Homozygous pxa1-1 aos double mutants were confirmed for pxa1 mutation by the pxa1 phenotype of failure to undergo successful seedling establishment without sucrose (Zolman et al., 2001) and for aos mutation by sterility phenotype of plants and PCR as described by Park et al. (2002). Homozygous cts-2 opr3-1 double mutants were confirmed for cts-2 mutation by PCR using CTS-specific 5’-CCAAAGCGCTGAAAGAAGA-GAGAT-3’ and T-DNA–specific primers JL202, 5’-5’-CATTTTATAA-TAACGCCGGAGCAGATC-3’, and for opr3-1 mutation by sterility phenotype of plants (Stintzi and Browse, 2000) and by PCR using OPF3-specific, 5’-TTCCTCTCAAGGACCATGCTC-3’, and T-DNA–specific primers, 5’-TCCTCAAATGGCGTTCTGTCAGTCTC-3’.

Germination Assays
Sterilized seeds (50 to 100 seeds) were imbibed on water agar plates (0.9% w/v) and incubated in a controlled environment growth cabinet under continuous light (150 mol m⁻² s⁻¹) at 20°C, except for experiments conducted for Figure 9 where temperatures of 27 and 31°C were used. Germination was scored as radicle emergence from the seed coat and endosperm. For stratification treatment, seeds were stratified at 4°C in dark for 3 d. The appropriate amount of JA, OPDA, or ABA was included in the water agar medium where germination assays were conducted in presence of JA, OPDA, and ABA.

Oxylipin Analysis
Oxylipin analysis was performed using a liquid chromatography–mass spectrometry (LC-MS) method described by Theodoulou et al. (2005). For developing seed analysis, developing seeds from 30 siliques were used. Briefly, 60 to 80 mg dry seed tissue was ground and extracted for 3 h at 4°C in 1.9 mL 70:30 acetone:50 mM citric acid with 20 ng prostaglandin A1 (Sigma-Aldrich) added as an internal standard. The tubes were left open in a fume hood to let the acetone layer evaporate overnight. Oxylipins were extracted from the aqueous phase by partitioning three times with diethyl ether, evaporating to dryness, and resuspending in 60% methanol prior to LC-MS analysis. Oxylipins were analyzed on an LCQ mass spectrometer (Thermo Separation Products) where separation was achieved on a LUNA 5 µm C18(2) 150 mm × 2 mm column (Phenomenex) using a gradient of mobile phases water + 0.2% formic acid and methanol + 0.2% formic acid with a flow rate of 0.4 mL/minute. LC-MS data were collected in full MS scan mode over the mass range m/z 8 to 500 in positive ionization mode. Oxylipins were quantified using response factors calibrated between internal standards and oxylipin standards. ABA, GA1, and GA4 were quantified using response factors calibrated between internal standards and oxylipon standards. ABA, GA1, and GA4 were purchased from Lew Mander (ANU, Canberra, Australia), and d6-ABA was purchased from ICON isotopes. ABA and GA4 were purchased from Sigma-Aldrich, whereas GA1 was a gift from Peter Hedden (Rothamsted Research, UK).

RNA Purification and cDNA Synthesis
Total RNA isolation from developing seeds was performed using solutions previously treated with diethyl pyrocarbonate to inhibit RNases. Approximately 200 seeds were ground with liquid nitrogen using a blue pestle. After homogenization, 150 µL of extraction buffer (0.2 M sodium borate decahydrate, 30 mM EGTA, 1% [v/v] SDS, and 1% [v/v] sodium deoxycholate), 10 mM DTT, 2% (w/v) polyvinyl pyrrolidone, and 1% (w/v) l-IGePal were added. After adding 6 µL of proteinase K (Roche Diagnostics), samples were mixed and incubated at 42°C for 90 min. After the incubation, 12 µL of 2 M KCl was added, and samples were mixed and incubated on ice for 60 min. To remove debris, samples were centrifuged at 15,000g for 20 min at 4°C. The supernatant was transferred to a fresh tube, and 54 µL of 8M LiCl was added. Samples were mixed and incubated at −20°C for 3 h. After the incubation, samples were centrifuged at 15,000g for 20 min at 4°C, and the RNA pellet was dissolved in 100 µL RNase-free water. RNA was purified with the RNeasy plant mini kit (Qiagen).

The quality of RNA was verified by demonstration of intact ribosomal bands following agarose gel electrophoresis in addition to the absorbance ratios (A260/280 and A260/230). Contaminating DNA was removed from RNA samples (1 µg) using the TURBO DNA-free kit (Ambion). First strand cDNA was synthesized from 0.5 µg DNA-free total RNA using the SuperScript III first-strand synthesis system (Invitrogen) with oligo (dT)20 primer, following the manufacturer’s instructions.

Affymetrix Genechip Experiment and Data Analysis
Isolated RNA was used for cDNA synthesis and biotin-modified RNA amplification using the MessageAmp III RNA amplification kit (Ambion). Three biological replicates per sample were hybridized independently to the Affymetrix ATH1 array, washed, stained, and scanned following the procedures described in the Affymetrix technical manual. The expression levels of genes were measured by signal intensities using the Micro Array Suite 5.0 software with a target signal of 500.

An R/bioconductor (Gentleman et al., 2004) package RP (Hong et al., 2006) was used to identify differentially expressed genes in a pairwise comparison of pxa1-1 and Col-0. Genes up- and downregulated in pxa1-1 developing seeds in comparison with Col-0 were ranked based on the combination of fold change and the consistency of the sample replications. A false positive predictions parameter (pfp) of 5% was used for the RP analysis, which gives the probability of detected genes being consistently selected by the RP method to be ~95%. See Supplemental Data Set 1 online for the top-ranked genes from the RP analysis.
The output of the RP pairwise comparison analysis was used to produce MIPS Functional Category (FunCat) Terms (Ruepp et al., 2004) (http://www.helmholtz-muenchen.de/en/mips/projects/functcat). Frequencies of upregulated and downregulated genes in pxa1-1 relative to the Col-0 wild type were given a FunCat group and compared with the frequency found for all genes represented on the array. A hypergeometric distribution P value was calculated for each FunCat group to show the significance of each group.

Quantitative RT-PCR Analysis

cDNA synthesized as described above was used. Real-Time PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and the MyIQ Real-Time PCR detection system (Bio-Rad) according to the manufacturer’s instructions. Expression of ACTIN was used for normalization. The primer sets used for PCR were as follows: DAD1F, 5'-AAACGTGCCTATGGTTTAC-3'; DAD1R, 5'-TCGACGTCTCATCTAACG-3'; ACX1F, 5'-TGCTTGCTTCTCTTTCTTCG-3'; ACX1R, 5'-TTAATGTGCATTGAAACGAA-3'; KAT2F, 5'-AGAGGACCAGCACAACAAA-3'; KAT2R, 5'-CAAGACTCAAGGCAAACCTGG-3'; GA2ox2F, 5'-CTCCGGCAGAGAAGAACAC-3'; GA2ox2R, 5'-CGTTGAGCTCTGGAATGTCA-3'; GA2ox6F, 5'-AGTGCGTCGTCGTCGAATAAGG-3'; GA2ox6R, 5'-AGCGAGTCACCAGCAATAC-3'; ACTIN, LH59 5'-TGAGAGATTCAGATGCCCA-3'; and LH40, 5'-TGATCCACGACGCTCTCAT-3'.

Three biological replicates were analyzed.

Protein Extraction and Immunoblot Analysis

Seeds of 3SS:HA-AB15 transgenic plants overexpressing AB15 with a hemagglutinin (HA) epitope tag (Lopez-Molina et al., 2002) were imbibed on water agar plates (0.9% w/v) containing ABA, OPDA, or ABA+OPDA (according to the treatment required) and stratified in the dark at 4°C for 3 d. Stratified seeds were transferred to a controlled environment growth cabinet (according to the treatment required) and stratified in the dark at 4°C for 2 s.

Following electrophoresis, proteins were transferred to nitrocellulose membranes (GE Healthcare), which were Ponceau S stained for assessment of equal loading. The membranes were then probed with a monoclonal anti-HA antibody (clone 3F10; Roche Applied Science) and subsequently with horseradish peroxidase-rabbit anti-mouse IgG (H+L) (Invitrogen). Immunoreactive bands were visualized with the ECL chemiluminescence reagent (GE Healthcare). Protein concentration was estimated according to Bradford (1976) with BSA as the standard.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ACT1 (At4g16760), ACX2 (At5g65110), AOS (At5g42650), AB15 (At2g36270), CO1 (At2g39940), CTS/PXA1 (At4g39850), DAD1 (At2g44810), GA2ox2 (At5g51810), GA2ox6 (At1g02400), KAT2 (At2g33150), LACS8 (At3g05970), LACS7 (At5g27600), and OPR3 (At2g06050).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. dnOPDA Abundance in Wild-Type, cts-2; and pxa1-1 Dry Seeds.

Supplemental Data Set 1. Rank Product Analysis of the Microarray Data Set.

Supplemental Data Set 2. Classification of Upregulated (620) and Downregulated (591) Genes in pxa1-1 According to the MIPS MATDB FUNCTIONAL Categories.

Supplemental Data Set 3. Expression of JA, GA, and ABA Metabolism and Signaling-Related Genes in the Microarray Experiment.

ACKNOWLEDGMENTS

We thank Bonnie Bartel, Alison Baker, John Browse, John Turner, Nam-Hai Chu, and Frederica Theodoulou for providing seeds of various mutants and transgenic lines. We thank Peter Hedden and Paul Staswick for providing us with GA1 and JA-ile standards, respectively. This work was supported by a UK Biotechnology and Biological Sciences Research Council (BBSRC) PhD studentship award to A.D. and BBSRC Grants BB/D006856/1 and BB/E022081/1.

Received November 20, 2010; revised January 21, 2011; accepted February 2, 2011; published February 18, 2011.

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12-Oxo-Phytodienoic Acid Accumulation during Seed Development Represses Seed Germination in Arabidopsis

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Plant Cell; originally published online February 18, 2011;
DOI 10.1105/tpc.110.081489

This information is current as of August 13, 2017

| Supplemental Data | /content/suppl/2011/02/22/tpc.110.081489.DC1.html |
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