Role of cis-12-Oxo-Phytodienoic Acid in Tomato Embryo Development[1[C][W]]

Stephan Goetz[2], Anja Hellwege[2], Irene Stenzel, Claudia Kutter, Valeska Hauptmann, Susanne Forner, Bonnie McCaig, Gerd Hause, Otto Miersch, Claus Wasternack[3], and Bettina Hause*

Department of Natural Product Biotechnology (present name: Department of Molecular Signal Processing; S.G., A.H., I.S., C.K., O.M., C.W.) and Department of Cell and Metabolic Biology (S.G., V.H., S.F., B.H.), Leibniz Institute of Plant Biochemistry, D–06120 Halle, Germany; Michigan State University, Department of Energy-Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824 (B.M.); and Biocenter, University of Halle, D–06120 Halle, Germany (G.H.)

Oxylipins including jasmonates are signaling compounds in plant growth, development, and responses to biotic and abiotic stresses. In Arabidopsis (*Arabidopsis thaliana*) most mutants affected in jasmonic acid (JA) biosynthesis and signaling are male sterile, whereas the JA-insensitive tomato (*Solanum lycopersicum*) mutant *jai1* is female sterile. The diminished seed formation in *jai1* together with the ovule-specific accumulation of the JA biosynthesis enzyme allene oxide cyclase (AOC), which correlates with elevated levels of JAs, suggest a role of oxylipins in tomato flower/seed development. Here, we show that *35S::SlAOC-RNAi* lines with strongly reduced AOC in ovules exhibited reduced seed set similarly to the *jai1* plants. Investigation of embryo development of wild-type tomato plants showed preferential occurrence of AOC promoter activity and AOC protein accumulation in the developing seed coat and the embryo, whereas 12-oxo-phytodienoic acid (OPDA) was the dominant oxylipin occurring nearly exclusively in the seed coat tissues. The OPDA- and JA-deficient mutant *spr2* was delayed in embryo development and showed an increased programmed cell death in the developing seed coat and endosperm. In contrast, the mutant *acx1a*, which accumulates preferentially OPDA and residual amount of JA, developed embryos similar to the wild type, suggesting a role of OPDA in embryo development. Activity of the residual amount of JA in the *acx1a* mutant is highly improbable since the known reproductive phenotype of the JA-insensitive mutant *jai1* could be rescued by wound-induced formation of OPDA. These data suggest a role of OPDA or an OPDA-related compound for proper embryo development possibly by regulating carbohydrate supply and detoxification.

Oxylipins are lipid-derived compounds such as jasmonic acid (JA), its precursor 12-oxo-phytodienoic acid (OPDA), and its metabolites like amino acid conjugates or hydroxylated derivatives. Oxylipins, preferentially JA and its Ile conjugate (JA-Ile), regulate various aspects of growth, development, and responses to biotic and abiotic stresses in plants (Wasternack, 2007; Browse and Howe, 2008; Howe and Jander, 2008; Browse, 2009a). All genes encoding enzymes of JA biosynthesis have been cloned from several plant species and the corresponding enzymes including isoforms have been characterized (Schaller and Stintzi, 2009). Enzymes active in OPDA formation such as a lipase, a 13-lipoxygenase, an allene oxide synthase, and an allene oxide cyclase (AOC) are located in the chloroplast, whereas OPDA reduction by the OPDA reductase3 (OPR3) and shortening of the carboxylic acid side chain by the fatty acid β-oxidation machinery takes place in peroxisomes. JA can be metabolized by several reactions. Among them conjugation with Ile catalyzed by JAR1, the JA amino acid conjugate synthase (Staswick and Tiryaki, 2004), leads to the most bioactive compound being crucial for the JA signaling cascade (Chung et al., 2009; Fonseca et al., 2009). All enzymes of jasmonate biosynthesis occur in resting, unstimulated cells. This allows JA formation within less than 5 min upon any stimulus that finally results in expression of JA biosynthesis genes and other JA-responsive genes (Glauser et al., 2009; Koo et al., 2009). The regulation of JA biosynthesis by substrate availability and by a positive feedback loop is well described by different assays including transgenic approaches (Laudert et al., 2000; Stenzel et al., 2003).

A role of jasmonates in wound- and herbivore-induced responses of leaves (for review, see Wasternack, 2007; Koo and Howe, 2009) and resistance to necrotrophic pathogens (Pieterse et al., 2009) is well documented.

[1] This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 363 project C5 and SFB 648 project C2 [to C.W. and O.M.] and project HA2655/12–1 [to B.H.]) and the Czech Academy of Sciences (grant no. KAN200380801).

[2] These authors contributed equally to the article.

[3] Present address: Laboratory of Growth Regulators, Palacky University, Slechtitelu 11, CZ–788 71 Olomouc, Czech Republic.

* Corresponding author; e-mail bhause@ipb-halle.de.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Bettina Hause (bhause@ipb-halle.de).

[C] Some figures in this article are displayed in color online but in black and white in the print edition.

[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.111.192658

 © 2012 American Society of Plant Biologists. All Rights Reserved.

Much less is known about the function of jasmonates in development such as that of flowers and seeds. Due to the relatively high JA levels in plant reproductive tissues, an essential role in flower development was assumed (Creelman and Mullet, 1997; Miersch et al., 1998). Indeed, the characterization of Arabidopsis (*Arabidopsis thaliana*) mutants defective in JA biosynthesis or perception revealed a role of jasmonates in pollen development of Arabidopsis: The coronatine- and jasmonate-insensitive mutant *coi1* (Xie et al., 1998) as well as the JA biosynthesis mutants *fad3-2 fad7-2 fad8* (McConn and Browse, 1996), *dad1* (Ishiguro et al., 2001), *opr3* (Stintzi and Browse, 2000), and *dde1* (Sanders et al., 2000) cannot produce viable pollen and are affected in stamen filament elongation. Therefore, all of them show an identical male-sterile phenotype (for review, see Wasternack and Hause, 2002; Browse, 2009b).

In tomato (*Solanum lycopersicum*), however, the function of JA in reproductive development differs from that in Arabidopsis. The JA-insensitive tomato *jai1* mutant, homologous to *coi1* in Arabidopsis, is female sterile due to a block in embryo development (Li et al., 2001, 2004). Pollen viability is also reduced in *jai1*, although there is enough fertile pollen for successful cross-fertilization to wild-type flowers (Li et al., 2004; B. McCaig, unpublished data). Although the degree of female sterility varies, at least one of the tomato mutants affected in JA biosynthesis also has reduced seed set. This concerns *spr2*, which is blocked at the plastidic ω-3-fatty acid desaturase FAD7 gene required for JA biosynthesis (Li et al., 2003), whereas *acx1a*, which is blocked in oxylipin β-oxidation during JA biosynthesis, is indistinguishable in seed set to wild type (Li et al., 2005). Under conditions that represent a generally low-stress environment insufficient to induce jasmonate production in wild-type plants, seed set of *spr2* plants is strongly affected, whereas *acx1a* plants exhibit still sufficient seed set. In contrast, field-grown *spr2* plants produce a higher number of seeds showing higher germination rates relative to seeds obtained from greenhouse-grown plants (Li et al., 2003, 2005). This suggests that seed set in *spr2* is influenced by maternal growing conditions and that stress conditions might result in residual levels of JA or other oxylipins sufficient for female fertility (Li et al., 2003, 2005).

A putative role of jasmonates in the development of female reproductive organs is suggested by the occurrence of AOC in ovules of developing wild-type flowers and the elevated levels of jasmonates in pistils (Hause et al., 2000; Stenzel et al., 2008). AOC catalyzes a crucial step in JA biosynthesis due to its enantiomer-specific catalysis and is encoded in tomato by a single-copy gene (Ziegler et al., 2000). Its preferential occurrence in ovules is reflected by the amount of OPDA, JA, and their amino acid conjugates and methyl esters, measured in pistils, all accumulating to high levels markedly exceeding that of leaves (Hause et al., 2000). The ovary-specific accumulation of jasmonates might result in expression of JA-induced genes, such as proteinase inhibitors (PIN2; Pena-Cortes et al., 1991), Leu amino peptidases (Fowler et al., 2009), endo-β-1,4-glucanase and γ-thionins (Milligan and Gasser, 1995), and defensins (Lay et al., 2003), all of them expressed in female flower organs. This suggests a specific role of JA and/or other oxylipins in ovules of tomato flowers.

The abundant accumulation of AOC protein in ovules, the preferential formation of oxylipins in pistils, and the female-sterile phenotype of *jai1* prompted us to analyze the role of AOC and oxylipins in tomato embryo development. This idea was initially supported by the fact that sterility of *jai1* may result from arrest in embryo/seed maturation, because *jai1* ovules have still the ability to be fertilized followed by production of small aborted seeds (Li et al., 2004). It has been discussed that this mutant seems to be affected in SlCOI1/JA-dependent supply of carbon or nitrogen by the maternal tissue thereby altering embryo development and/or generation of vitamin(s) or cofactor(s) that requires transport from maternal tissues to the developing embryo (Li et al., 2004). This might be similar to the role of biotin synthesis in Arabidopsis embryogenesis (Patton et al., 1998). Furthermore, *jai* flowers are defective in the formation of JA-inducible secondary metabolites such as caffeoylputrescine (Chen et al., 2006).

Seed development in plants starts after double fertilization, leading to formation of the diploid embryo and the triploid endosperm, which nourishes and protects the developing embryo (Dumas and Rogowsky, 2008). The developing embryo and the endosperm are surrounded by the maternal tissue of the ovule, the nucellus, and the integuments, which are later transformed into the seed coat. Thus, the proper development of the embryo is influenced by tissues exhibiting potentially different genomes: the diploid embryo (haploid maternal and haploid paternal genomes), triploid endosperm (diploid maternal and haploid paternal genome), and diploid maternal tissue. Moreover, during seed growth, coordination is necessary between these three types of tissues. In Arabidopsis an interplay between embryo endosperm and developing seed coat during growth was shown to be essential for seed size determination (Berger et al., 2006).

To elucidate the putative function of jasmonates in embryo development, we generated *35S::SlAOC-RNAi* plants and compared their phenotype to *jai1*. *SlAOC* promoter activity and AOC protein accumulation was analyzed in developing wild-type seeds and isolated embryos. Under the growth conditions used in our experiments, the *spr2* mutant was found to be delayed in embryo development, whereas the *acx1a* mutant exhibited normal seed formation as previously published (Li et al., 2005). Therefore, we analyzed the oxylipin profile of developing wild-type seeds and programmed cell death (PCD) of developing wild-type and *spr2* seeds. The delay of embryo development in *spr2* plants was accompanied by an increased PCD in the developing seed coat and endosperm. Since

OPDA was the major oxylipin occurring nearly exclusively in the seed coat of developing wild-type seeds, rescue of seed formation in the JA-insensitive mutant *jai1* was performed by wound-induced OPDA formation. The data suggest a role of OPDA or a related compound originating from the maternal tissues for proper embryo development.

RESULTS

35S::*SlAOC-RNAi* Lines Exhibit a *jai1*-Similar Embryogenic Phenotype

We previously generated *35S*::*SlAOC*-sense and -antisense lines to modulate JA levels in tomato. An up-regulation and down-regulation, respectively, of the *SlAOC* transcript was found in leaves (Stenzel et al., 2003). The SlAOC protein level was not diminished in ovules of antisense lines compared to wild-type ovules (Miersch et al., 2004). This prompted us to generate *35S*::*SlAOC-RNAi* lines. Among 48 independent lines about 40 lines showed reduced *SlAOC* transcript (Supplemental Fig. S1A) and protein (Supplemental Fig. S1B) levels in leaves accompanied by decreased levels of OPDA and JA in unwounded leaves and more pronounced in wounded leaves (Supplemental Fig. S1C). Accordingly, ovules of developing flower buds showed nearly undetectable levels of SlAOC protein by immunocytochemical analysis of the RNAi lines (Fig. 1, C versus A), which was similar to that of the sections treated with preimmune serum (Fig. 1, A′–C′). This is different from *jai1* plants, which showed SlAOC protein in ovules as well (Fig. 1B), pointing to a developmental regulation of *SlAOC* expression. Less than 5% of hand-pollinated flowers of RNAi lines developed into fruits (Fig. 1D), but these did not contain mature seeds (not shown). Even upon pollination of *35S*::*SlAOC-RNAi* flowers with wild-type pollen, fruits were developed, but an arrest in seed development appeared. Most traits in the flower phenotype, such as size and number of petals or sepals were not altered. The arrest in seed development, however, is reminiscent to that of the female-sterile *jai1* mutant (Li et al., 2001, 2004). The limited seed set of the *35S*::*SlAOC-RNAi* lines did not allow a detailed analysis of developing seeds. Therefore, the phenotype of *jai1* was analyzed cytologically by comparing developing seeds of wild-type plants (Fig. 2, A and B) and of *jai1* mutant plants, either fertilized with wild-type pollen (Fig. 2, C and D) or self-fertilized (Fig. 2, E and F). Developing wild-type seeds are characterized by a well-developed embryo enclosed by endosperm and the developing seed coat. In contrast, embryos of *jai1* plants were smaller and appeared poorly developed. Most obvious, the endosperm tissue was collapsed and seemed to be separated from the developing seed coat tissues (Fig. 2, C–E). The arrested embryo development was visible in *jai1* plants fertilized with *jai1* pollen, but also with wild-type pollen. This led to the conclusion that embryo development in tomato might be regulated by JA or related oxylipins synthesized and/or perceived in maternal tissues.

Early Stages of Embryo Development Exhibit *SlAOC* Promoter Activity and SlAOC Protein Accumulation in the Embryo and the Developing Seed Coat

The similarities between the reproductive phenotype of *35S*::*SlAOC-RNAi* lines and *jai1* plants led us to inspect *AOC* promoter activity and AOC protein accumulation, an essential enzyme in JA biosynthesis.

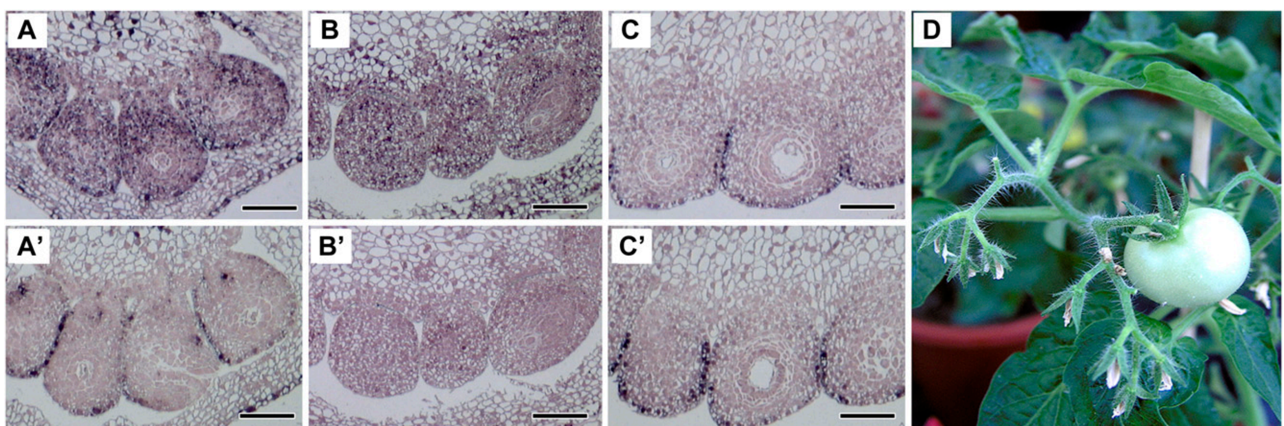

Figure 1. AOC protein occurs specifically in ovules of *jai1* and wild-type plants, but diminished in *35S*::*SlAOC-RNAi* lines, which are blocked in seed development. Immunocytochemical detection of SlAOC protein with an anti-SlAOC antibody in cross section of unpollinated flowers of 6-week-old plants of the wild-type MicroTom (A), *jai1* (B), and a *35S*::*SlAOC-RNAi* line (C). The corresponding controls with the preimmune serum are shown in A′ to C′. Beside a cross reactivity of the epidermal layer AOC protein is visible within ovules of wild type and *jai1*, but much less AOC protein was detected in ovules of the *35S*::*SlAOC-RNAi* lines. In D the strong flower phenotype of one of the *35S*::*SlAOC-RNAi* lines is shown. Here, most of the hand-pollinated flowers are blocked in development. The bars represent 50 μm (A–C′). [See online article for color version of this figure.]

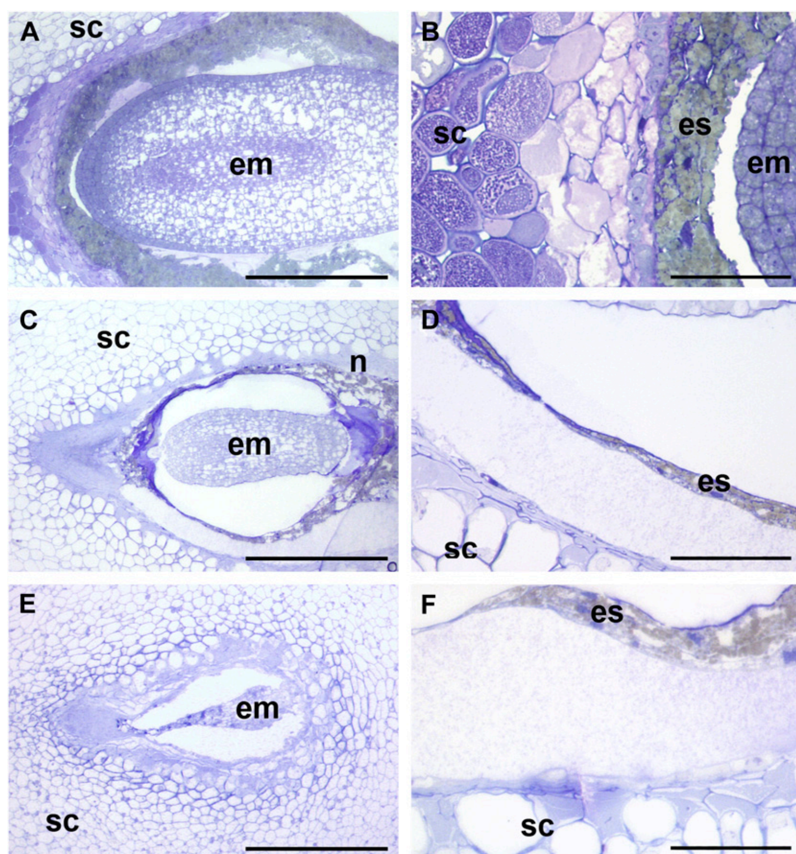

Figure 2. Seed development in the mutant *jai1* is affected in endosperm and embryo morphology. Seeds (15 DAP) of wild type (cv MicroTom; A and B) and *jai1* mutant plants (C–F), either fertilized with wild-type pollen (C and D) or self-fertilized (E and F), were processed for histological analyses as described in "Materials and Methods." Longitudinal sections were stained with toluidine blue. Note the collapsed endosperm and the small embryo in seeds of the *jai1* mutant. sc, Developing seed coat; es, endosperm; em, embryo. Bars represent 200 μm in A, C, and E, and 50 μm in B, D, and F. [See online article for color version of this figure.]

Transgenic lines carrying a truncated *SlAOC* promoter upstream of the GUS reporter gene were generated and characterized elsewhere (Stenzel et al., 2008). With these lines *SlAOC* promoter activities tested during vegetative development and in response to environmental stimuli were identical to that detected in transgenic lines carrying the full-length promoter.

Here, developing seeds and embryos were analyzed in crosses *SlAOC*::*GUS* × *SlAOC*::*GUS* (Fig. 3A, top row for seeds, bottom row for isolated embryos) and wild type × *SlAOC*::*GUS* (Fig. 3A, middle row). GUS staining in intact developing seeds indicated strong *SlAOC* promoter activity from 10 d after pollination (DAP) onwards in the developing seed coat. The localized staining in embryos of seeds of wild-type plants fertilized with *SlAOC*::*GUS* pollen indicates that GUS staining originates also from filial tissue (Fig. 3B). This is supported by the fact that embryos at globular and torpedo stage, all isolated before staining, exhibited *SlAOC* promoter activity. Absence of *Sl*AOC promoter activity from the endosperm could not be validated by GUS staining because of a possible diffusion of the dye from tissue exhibiting high GUS activity. In seed cross sections of different developing stages, however, immunocytochemical analysis indicated SlAOC protein in the embryo and the developing seed coat, but not in the endosperm (Fig. 3B), indicating that the *SlAOC* promoter activity visualized by GUS staining corresponds to AOC protein accumulation spatially and temporally. The strongest fluorescent signal indicative for occurrence of AOC protein appeared in embryos; the chalazal part of the seed showed strong staining as well (Fig. 3B). Therefore, both organs, the embryo and the developing seed coat, may have the capacity to synthesize JA.

A clearly detectable amount of AOC protein during any developmental stages is usually accompanied by elevated levels of JA, leading to accumulation of transcripts of JA-responsive genes, such as *PIN2* (Wasternack, 2007). Transcripts of *PIN2* were detectable by in situ hybridization in ovaries of flower buds, an organ unequivocally shown to contain high levels of jasmonates (Hause et al., 2000), but also in developing embryos of wild-type plants (Supplemental Fig. S2).

Embryos of the *spr2* Mutant Are Delayed in Development, and *spr2* Mutant Seeds Show Enhanced PCD in Developing Seed Coat and Endosperm

To validate the still correlative link between AOC occurrence, JA generation, and embryo development drawn from data obtained from wild-type plants, we analyzed the JA-deficient mutant *spr2*. This mutant was shown to be impaired in wound-induced JA accumulation and the production of the transmissible wound signal due to a defect in the plastid-located fatty acid desaturase that catalyzes the ω-3-desaturation of linoleic acid to the JA precursor, α-linolenic acid (α-LeA; Li

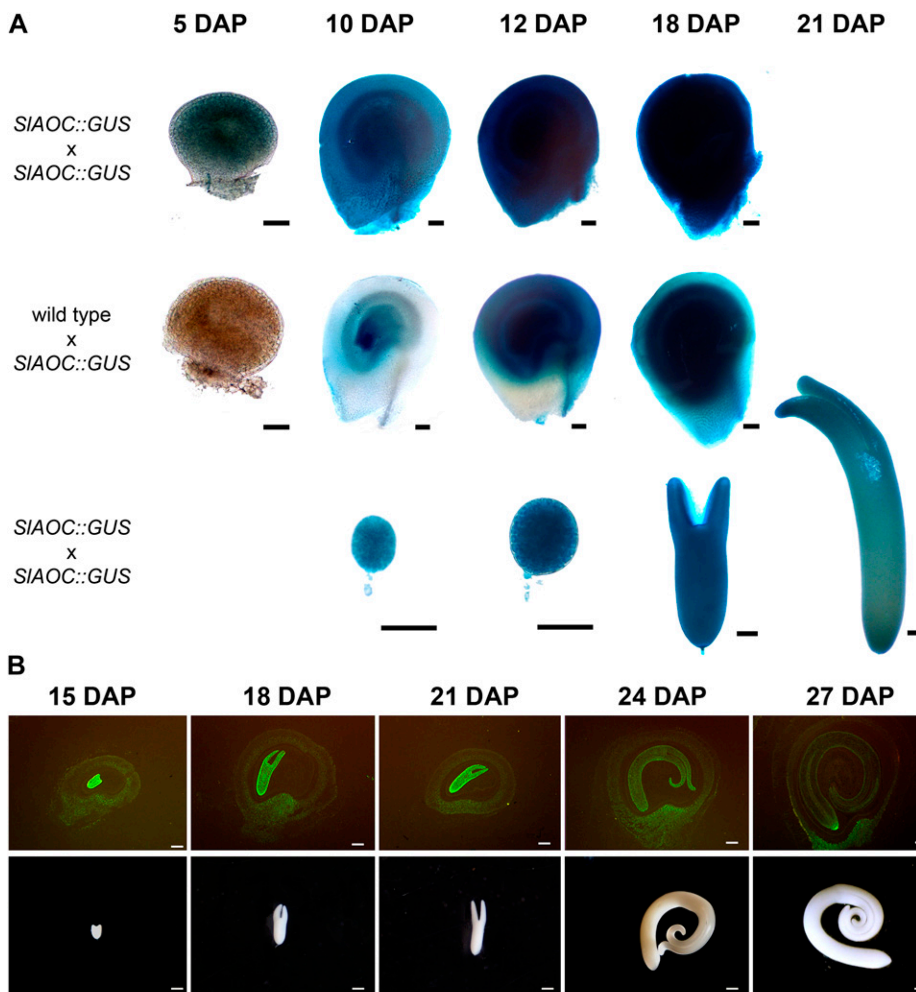

Figure 3. *SlAOC* promoter activity and SlAOC protein accumulation occur in early stages of tomato embryo development. A, High *SlAOC* promoter activity visualized by GUS staining appears in the globular (10 and 12 DAP) and the torpedo (18 and 21 DAP) stages of embryo development. In the top and the middle row complete seeds of self-fertilized *SlAOC*::*GUS* plants (MicroTom background) and wild type fertilized with pollen of *SlAOC*::*GUS* plants, respectively, are shown. In the bottom row isolated GUS-stained embryos of self-fertilized *SlAOC*::*GUS* plants are shown. B, Immunocytochemical detection of SlAOC protein in cross sections of seeds of the wild type (top row). The green fluorescence indicates occurrence of SlAOC protein in the embryo of the heart (15 DAP), the torpedo (18 and 21 DAP), the walking stick (24 DAP), and the curled cotyledon (27 DAP) stage, in the developing seed coat of each corresponding stage, but not in the endosperm. The bottom row shows isolated embryos of the corresponding stage. Bars represent 100 μm in A, and 250 μm in B.

et al., 2003). Consequently, wounded *spr2* leaves contain only about 12% JA level from that of wounded wild-type leaves (Li et al., 2002), which is similar to that of *spr2* flowers compared to the wild type and may attribute to the fertility of *spr2* plants (Li et al., 2003). Here, we show, however, that the embryo development is also affected in *spr2* plants. Under controlled greenhouse conditions wild-type seeds carried embryos in the curled cotyledon stage at 30 DAP (Fig. 4A), while embryo development of *spr2* plants was delayed, showing embryos in the torpedo stage 30 DAP (Fig. 4D). Among 19 different *spr2* plants and a total number of 436 inspected developing seeds about 70% exhibited this delay in embryo development, and most fruits did not contain mature seeds. In contrast, field-grown *spr2* plants exhibited higher seed set (G. Howe, personal communication).

To characterize seed development of *spr2* plants in comparison to wild-type plants, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) analyses were performed to visualize cells undergoing PCD. Using this method, nonrandom, internucleosomal fragmentation of nuclear DNA becomes visible, which is one of the basic features of PCD and occurs as a result of specific endonuclease activation prior to condensation of nuclear chromatin (Greenberg, 1996). In positive controls generated by treatment of sections with DNase I to achieve appropriate fragmentation, all nuclei of a section showed TUNEL-positive signals (Supplemental Fig. S3). In cross sections of wild-type seeds being in preceding stages of embryo development, few positive TUNEL signals occurred nearly exclusively in the developing seed coat and the endosperm of the wild-type seeds, whereas no signal was detectable in embryo tissue (Fig. 4B). Embryo tissue of *spr2* mutant also did not show label, but the developing seed coat and endosperm of *spr2* mutant seeds exhibited a significantly higher number of positive TUNEL signals than the wild type (Fig. 4E). The concomitant 4,6-diamidino-phenylindole (DAPI) staining (Fig. 4, C and, F) clearly indicates that a similar number of nuclei were visible in cross sections of endosperm and seed coat tissues of both plant genotypes, thereby showing that the difference in the number of TUNEL- positive signals was not due to different numbers of nuclei analyzed. These data suggest that JA or any other oxylipin formed downstream of α-LeA is necessary for seed coat and endosperm function

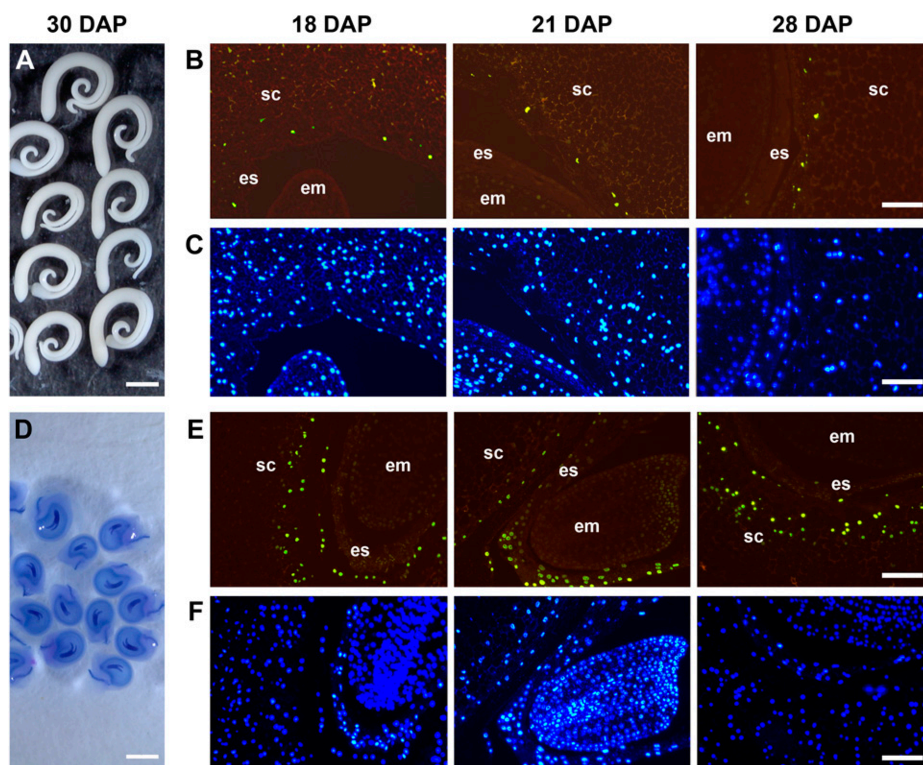

Figure 4. Embryos of the *spr2* mutant are blocked in development and exhibit enhanced PCD in the developing seed coat and endosperm. At 30 DAP, embryos of the wild-type Castlemart were in the curled cotyledon stage (A), whereas embryos of the *spr2* mutant were arrested in development (D). PCD was analyzed during development of seeds of the wild type (B and C) and the *spr2* mutant (E and F). Cross sections of the torpedo (18 and 21 DAP) and the curled cotyledon (28 DAP) stage were analyzed by the TUNEL assay (B and E) and concomitant DAPI staining (C and F). The wild-type tissues show few TUNEL-positive signals only (B). In contrast, a high number of TUNEL-positive signals appeared nearly exclusively in the seed coat tissues and in the endosperm of the *spr2* mutant (E), but not in the embryo. DAPI staining of the corresponding cross sections of the wild type (C) and *spr2* mutant (F) indicates the number of nuclei. sc, Developing seed coat; es, endosperm; em, embryo. Bars represent 1 mm in A and D, and 100 μm in B, D, E, and F.

and might have a role to avoid PCD in endosperm tissue, thereby leading to proper embryo development.

The JA-Deficient and OPDA-Forming *acx1a* Mutant Exhibits Embryo Development Similar to the Wild Type

The *spr2* mutant is affected in ω-3-fatty acid desaturase activity (Li et al., 2003). Consequently, other oxylipins besides JA might be also diminished, thus being responsible for the embryogenic phenotype of *spr2* plants. To confine the number of putative oxylipins downstream of the ω-3-fatty acid desaturase reaction, we inspected *acx1* plants. This mutant is affected in *ACX1A*, one of the two genes known so far that encode acyl-CoA oxidases in tomato (Li et al., 2005). ACX1A catalyzes the first step in peroxisomal fatty acid β-oxidation and is involved in JA biosynthesis. Wild-type embryos in the curled cotyledon stage (Fig. 5A) at 30 DAP were phenotypically similar to *acx1a* mutant embryos (Fig. 5B), thereby correlating with the previous observation that *acx1a* plants did not show a diminished seed production (Li et al., 2005). Inspection of cross sections of seeds at 30 DAP did not show differences between tissues of the seed coat, endosperm, and embryo of the wild type (Fig. 5C) and the *acx1a* mutant (Fig. 5D). These data suggest that an oxylipin formed within the lipoxygenase pathway downstream of α-LeA and upstream of JA is necessary and sufficient for proper embryo development. Seeds from *35S::AOC-RNAi* lines aborted during development as described above. This raises the question whether a product generated downstream of AOC but prior to ACX1A, such as OPDA, is likely to be essential for seed development.

OPDA Is the Most Abundant Oxylipin and Accumulates Nearly Exclusively in the Seed Coat Tissues

To analyze whether OPDA might play a role for proper seed development, we quantified OPDA and several jasmonates in intact developing seeds, in isolated seed coats and embryos at 27 DAP, and in developing fruits of wild-type plants (Fig. 6). Indeed, OPDA was the most prominent oxylipin detectable in seeds from 15 to 27 DAP (Fig. 6A). Dinor-OPDA, JA,

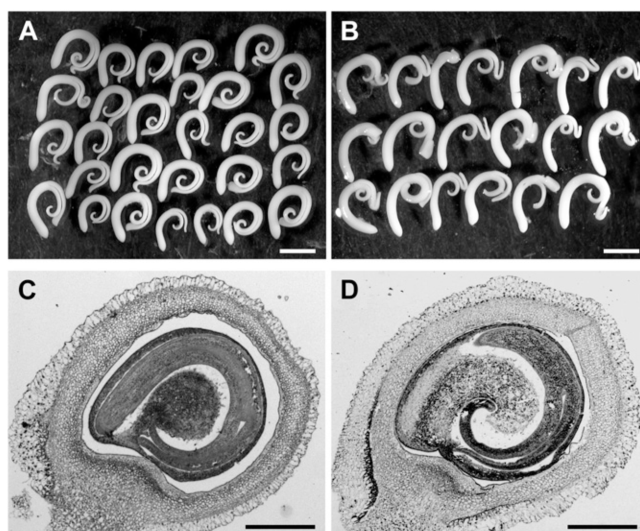

Figure 5. The JA-deficient and OPDA-forming *acx1a* mutant exhibits an embryo development similar to the wild type. Embryos from wild-type (A) and mutant *acx1a* (B) were prepared from seeds at 30 DAP and showed all the curled cotyledon stage. The cross sections of wild-type (C) and mutant (D) seed stained with toluidine blue did not show differences in morphology. Bars represent 1 mm in A and B, and 100 μm in C and D.

11-OH-JA, and 12-OH-JA were detected only at negligible levels. After mechanical dissection of wild-type seeds at 27 DAP into seed coat and embryo, OPDA was found preferentially in the seed coat tissues and to low amount only in the embryo (Fig. 6B). An equivalent analysis of oxylipin levels in seed coat tissues and embryos of *spr2* seeds could not be done due to their arrest in embryo development. However, in the few *spr2* seeds generated we measured an OPDA level in the range of about 10% of that detected in wild-type seeds (Table I). We tried to rescue the reproductive phenotype of *spr2* plants by daily injection of 200 μL of a 100 μM OPDA solution into fruits between 15 and 21 DAP. A rescue appeared only occasionally to less than 10% obviously due to metabolic conversion of OPDA to JA by the enzymatic steps downstream of the ω-3-fatty acid desaturase being active in the *spr2* mutant. More than 90% of the injected OPDA were found to be converted to JA and 12-hydroxy-JA. This conversion was similarly recorded upon injection of D_5-OPDA, resulting in deuterated JA and deuterated 12-hydroxy-JA. This corresponds to the strong shift into JA and 12-hydroxy-JA upon wounding of tomato leaves (Miersch et al., 2008). Nevertheless, the results shown in Figure 6 and Table I demonstrate that OPDA is the major oxylipin in developing seeds and accumulates preferentially in the seed coat tissues.

Properties of *acx1a* and *jai1* Plants: A Role of OPDA in Embryo Development?

The delayed embryo development in *spr2* seeds (Fig. 4D), and the normal embryo development in the JA-deficient and OPDA-forming *acx1a* mutant as well as the nearly exclusive accumulation of OPDA in the developing seed coat prompted us to analyze levels of OPDA, JA, and JA-Ile in developing seeds of wild type, *acx1a*, and *spr2*. In complete wild-type seeds (cv Castlemart) high levels of OPDA, JA, and JA-Ile were found (Table I). The corresponding amounts in *acx1a* seeds were similar for OPDA, and levels of JA and JA-Ile were lower than in wild type, but still measurable (Table I). These data indicate the expected accumulation of OPDA and deficiency in JA/JA-Ile in *acx1a* seeds. There is, however, like in *acx1a* leaves (Li et al., 2005), a residual amount of JA/JA-Ile that may attribute to the

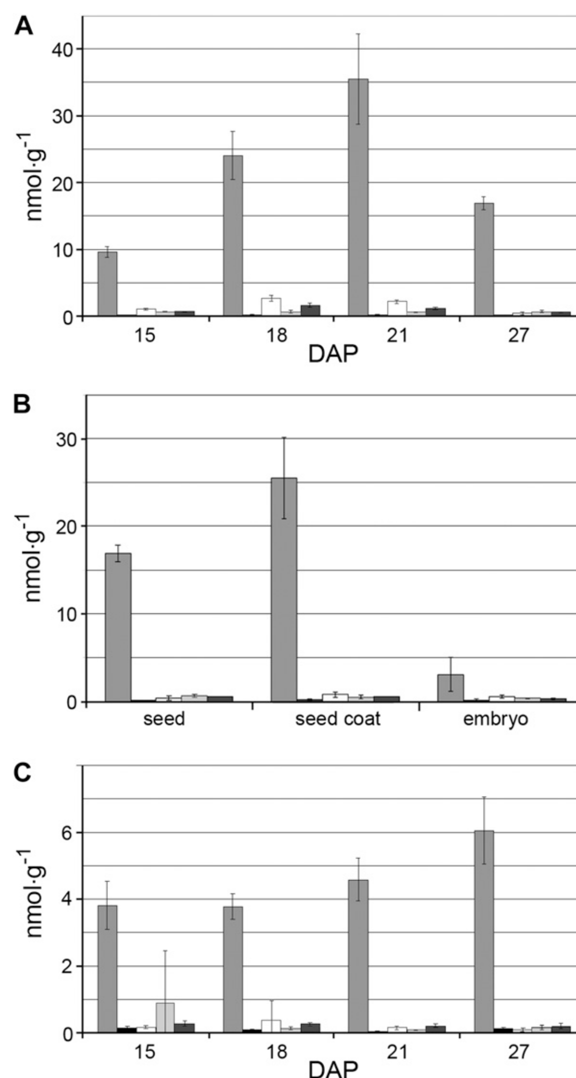

Figure 6. Oxylipin accumulation in seeds and fruits of wild-type tomato. Seeds (A) and fruits (C) of wild-type plants were collected at the time points indicated, and seeds at 27 DAP were mechanically dissected into seed coat and embryo (B). Content of OPDA (gray), dn-OPDA (black), JA (white), 12-OH-JA (light gray), and 11-OH-JA (dark gray) were determined as described in "Material and Methods." OPDA is the most prominent compound in developing seeds, thereby accumulating nearly exclusively in the seed coat as shown for 27 DAP (B).

Table I. *Levels of OPDA, JA, and JA-Ile and transcript accumulation of SlACX1A and SlACX1B in seeds of wild type (Castlemart), spr2, and acx1a at 21 DAP*

Genotype	OPDA[a]	JA[a]	JA-Ile[a]	*ACX1A*[b]	*ACX1B*[b]
Wild type	3,298 ± 1,029	393 ± 183	183 ± 540	0.443 ± 0.097	$8 \cdot \times 10^{-5} \pm 5 \times 10^{-5}$
spr2	188 ± 178	29 ± 16	n.d.[c]	n.d.[c]	n.d.[c]
acx1a	5,224 ± 815	67 ± 41	16 ± 9	0.426 ± 0.030	$20 \cdot \times 10^{-5} \pm 9 \times 10^{-5}$

[a]Mean ± SD, given in pmol/g fresh wt, $n = 6$. [b]Mean ± SD of relative expression levels in relation to the reference gene *SlTIP4l*, $n = 3$. [c]Not determined.

normal embryo development of *acx1a* mutant. The few *spr2* seeds generated contained less than 10% OPDA and JA compared to the wild type, thus corresponding to the above-mentioned percentage for *spr2* flowers (Li et al., 2003).

To test putative contribution of the two *ACX1* family members, expression of *ACX1A* and *ACX1B* was recorded by quantitative reverse transcription (RT)-PCR (Table I). Both genes were expressed in developing seeds of wild type and *acx1a*. The relative transcript accumulations of *ACX1B* were, however, about 3 orders of magnitude lower than those of *ACX1A* in both genotypes. Consequently, a contribution of *ACX1B* on the observed residual JA formation in *acx1a* mutant is highly unlikely.

To find out whether the normal embryo development in *acx1a* mutant was caused by the residual amount of JA/JA-Ile or by elevated levels of OPDA, we analyzed *jai1* plants. The lack of JA/JA-Ile perception in this mutant (Li et al., 2004; Yan et al., 2009; Sheard et al., 2010) should allow to test OPDA activity independently of JA/JA-Ile by injection of OPDA into fruits. Since the success of such a treatment, however, might be dependent of the right time window and position for application, we took advantage from the known wound-induced generation of OPDA and JA/JA-Ile (Fig. 7). Although genes encoding JA biosynthetic enzymes are known to be regulated by COI1/JA (Reymond et al., 2000), their developmentally regulated occurrence in flower organs of *jai1* (Fig. 1; Miersch et al., 2004) allows to induce elevated OPDA and JA levels by wounding. Beginning at the onset of flowering, all leaves of *jai1* plants were daily wounded by squeezing with a forceps, and subsequently mature fruits were checked for the occurrence of seeds. In nonwounded *jai1* plants all fruits tested were without any seeds ($n > 60$, example shown in Fig. 7A). Daily repeated wounding of leaves led to generation of seeds in 20 out of 23 fruits checked. The seed number varied from one seed per fruit up to 25 seeds per fruit, resulting in a mean of 5.87 ± 5.79 seeds per fruit ($n = 23$, example shown in Fig. 7B). To check whether leaf wounding resulted in an increase of OPDA in fruits, OPDA levels were determined from fruits of nonwounded and repeatedly leaf-wounded *jai1* plants (Fig. 7C) and compared with fruits of nonwounded wild-type plants (Fig. 6C). Young, green fruits of a size of 3- to 6-mm diameter were collected from five *jai1* plants each and subjected to OPDA determination. Although not statistically significant ($P = 0.06$, Student's t test), *jai1* fruits of wounded plants contained more OPDA than fruits of nonwounded plants, whereas nonwounded wild-type fruits contained about 5-fold-higher OPDA level than unwounded *jai1* fruits as expected (Fig. 6C versus 7C). This suggests that the reproductive *jai1* phenotype was rescued by endogenously formed OPDA or an OPDA-like compound. Putatively formed JA and JA-Ile are excluded to be active compounds due to the block in JA/JA-Ile perception (Li et al., 2004).

DISCUSSION

A role of jasmonates in flower development was initially shown by identification of Arabidopsis mutants affected in JA biosynthesis such as *dad1*, *fad 3-2 fad 7-2 fad 8*, *allene oxide synthase*, and *opr3* or in JA signaling such as *coi1-1* and *jin1* (Browse, 2009b). Most of them are male sterile due to diminished stamen filament elongation and loss of viable pollen. In case of loss of function of the F-box protein COI1 there is a functional diversity between Arabidopsis and tomato.

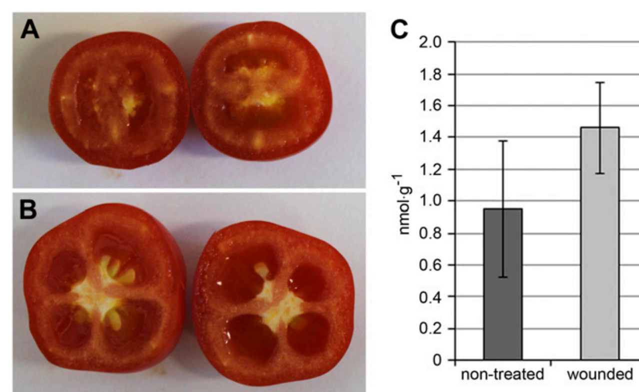

Figure 7. Seed set and OPDA content in fruits of *jai1* plants after leaf wounding. Mature fruit of nontreated (A) and wounded (B) plants as well as OPDA levels in green fruits harvested from nontreated and wounded plants (C). Daily leaf wounding performed for 5 d after onset of flowering resulted in formation of seeds and enhanced OPDA levels in *jai1* fruits. For comparison of OPDA content of nonwounded wild-type fruits see Figure 6C. In C the mean and SD of five independent determinations are shown. [See online article for color version of this figure.]

In contrast to the exclusive male sterility of *coi1* plants of Arabidopsis (Xie et al., 1998), the *jai1* plants affected in the tomato homolog of COI1 are less affected in pollen viability, but are additionally female sterile (Li et al., 2004). The female-sterile phenotype of *jai1* plants might be caused by a diminished expression of JA-responsive genes, and including JA biosynthesis genes (Li et al., 2004), since latter are expressed in a COI1-dependent manner (Reymond et al., 2000; Devoto et al., 2005). This might result in two simultaneous effects, diminished biosynthesis of JAs and related oxylipins as well as the loss of JA-responsive gene products.

Wild-type tomato plants show a preferential accumulation of AOC protein in ovules and elevated levels of JA and other oxylipins in the ovule-containing pistils of flower buds (Fig. 1; Hause et al., 2000). Similarly, the AOC gene is up-regulated in pollinated flowers (Wang et al., 2009). These data and the female-sterile phenotype of *jai1* plants prompted us to investigate the role of JA in embryo development. Therefore, we generated *35S::SlAOC-RNAi* lines that showed an early flower abortion and an impairment in seed development (Fig. 1). Cytological analysis of seeds of *35S::SlAOC-RNAi* plants could not be performed due to this strong phenotype. By analogy, however, a cytological comparison of wild-type and *jai1* plants (Fig. 2) was done because the phenotype of *35S::SlAOC-RNAi* plants is reminiscent to *jai1* plants. Seeds of *jai1* plants showed small and poorly developed embryos.

In tomato AOC is encoded by a single-copy gene (Ziegler et al., 2000) with high promoter activity in young seeds, vascular bundles, and green fruits (Stenzel et al., 2008). There is a close correlation between AOC promoter activity, AOC protein accumulation, and JA levels in different developmental stages of tomato and in response to various environmental stimuli (Hause et al., 2000; Stenzel et al., 2003, 2008). Similarly, AOC promoter activity and AOC protein accumulation closely correlated from heart stage of embryo development onwards (Fig. 3). The AOC protein accumulated in the embryo and developing seed coat, but not in the endosperm. The levels of oxylipins, however, accumulated with a peak at 21 DAP nearly exclusively in the seed coat tissues. The JA precursor OPDA was the dominant compound detectable in wild-type seeds (Fig. 6). This suggests that decreased formation of OPDA or a OPDA-like compound in the *35S::SlAOC-RNAi* plants attributes to the observed embryogenic phenotype in these transgenic lines. At least for wounded leaves strongly decreased levels of JA and OPDA in the transgenic lines could be shown (Supplemental Fig. S1). Obtaining similar data for the seeds of the *35S::SlAOC-RNAi* plants was impossible due to the low seed set. Therefore, we inspected *spr2* plants that are affected in the formation of α-LeA. Under controlled conditions preventing environmental stress, *spr2* plants showed a delay in embryo development thus supporting the assumption that any compound generated downstream of α-LeA is necessary for proper embryo development. The residual amount of JA detected in *spr2* flowers (18% of the wild type) was assumed to be sufficient to promote fertility (Li et al., 2003). OPDA levels were not checked. Since *spr2* plants are affected in the plastid ω-3-fatty acid desaturase, they should have JA and OPDA deficiency. Indeed, we could detect much less amounts of both compounds in the few *spr2* seeds generated. Consequently, the observed delay in development of *spr2* embryos might be caused by deficiency in JA and/or OPDA. The delay led to impaired seed development, resulting in about 30% developed seeds in comparison to wild type. This reduced seed development of *spr2* plants appeared under greenhouse conditions used here. In field-grown *spr2* plants, however, where stress-induced generation of oxylipins may occur, a significantly higher seed set was observed (G. Howe, personal communication). This is consistent with the assumption that for seed development a threshold of JA and/or OPDA is necessary. Regulation of plant development by a threshold of signaling compounds is a common phenomenon (Boss et al., 2010).

Taking into account the *35S::SlAOC-RNAi* plants, compounds essential for embryo development could be narrowed down to any compound downstream of the AOC-catalyzed step. The normal seed development, however, in *acx1a* plants, which showed elevated levels of OPDA but diminished JA levels, suggests that OPDA and/or its metabolites are active in normal embryo development. The *acx1a* embryos were similar under our growth conditions to the wild type in all developmental stages as exemplified and shown for the curled cotyledon stage (Fig. 5). Cross sections of seeds in the curled cotyledon stage showed no differences in morphology of seed coat, endosperm, and embryo between wild type and *acx1a*. An interpretation, however, of the *acx1a* embryo phenotype in terms of preferential role of OPDA, is hampered by the fact that *acx1a* seeds showed a residual level of JA/JA-Ile that corresponds to data for leaves (Li et al., 2005). It is unlikely that the second *ACX1* gene, *ACX1B*, attributes to this residual amount due to (1) its very low expression compared to that of *ACX1A* in the *acx1a* mutant and (2) the preference of ACX1A for C18 cyclopentanoid-CoAs that are the required JA precursors (Li et al., 2005). It cannot be excluded so far that additional ACX genes homologous to *AtACX1*, *AtACX3*, and *AtACX4* and detected in the tomato EST database may code for enzymes active in JA biosynthesis (Li et al., 2005).

Like the *spr2* plants, which show residual amount of JA/JA-Ile and partial fertility (Li et al., 2003), residual amount of JA/JA-Ile in *acx1a* plants may account for the observed proper embryo development. The following observations, however, make an exclusive activity of JA/JA-Ile in embryo development highly improbable: (1) the defect in embryo development of *jai1* could be rescued by wound-induced endogenous formation of OPDA, and (2) although both *spr2* and

acx1a plants have residual levels of JA/JA-Ile, only *acx1a* plants exhibiting elevated OPDA levels showed normal embryo development. Obviously, *acx1a* plants have the required threshold of OPDA. In contrast, *jai1* plants do not seem to have a sufficient amount of OPDA without additional stress treatment. In nonstressed *jai1* plants, missing JA perception may lead to diminished OPDA levels, since the well-known positive feedback of JA/JA-Ile on oxylipin biosynthesis cannot be active (Wasternack, 2007). Only a stress treatment, such as repeatedly performed wounding, causes an OPDA accumulation sufficient for seed formation in *jai1* fruits. To date, there is no hint how OPDA is perceived, but its perception via the JA receptor/coreceptor complex can be clearly excluded (Thines et al., 2007; Katsir et al., 2008; Fonseca et al., 2009; Yan et al., 2009; Sheard et al., 2010).

It is worthwhile to mention, although not directly comparable, that in the moss *Physcomitrella patens*, which is able to form OPDA but not JA, the *aoc* knockout plants of *P. patens* exhibit reduced fertility (Stumpe et al., 2010). Except the membrane-bound OPDA occurring exclusively in Arabidopsis species and forming the so-called Arabidopsides, there is no report so far on OPDA metabolites, such as amino acid conjugates. A recent inspection of *P. patens* (Stumpe et al., 2010) and some higher plant species (R. Kramell, personal communication) failed to detect such OPDA derivatives.

The correlative data given here for OPDA raise the question, which processes might be affected by OPDA in the well-documented communication between the maternal tissues, such as integument and nucellus, and the filial tissues, such as endosperm and embryo. This repeatedly described communication is necessary for proper seed development and involves auxin and ethylene as preferential signals (Balbi and Lomax, 2003; Wang et al., 2009). It might be, however, negatively affected by insufficient levels of OPDA. This assumption is supported by the fact that developing *spr2* seeds exhibited increased PCD in the developing seed coat and endosperm (Fig. 4). Since seed coat tissues and endosperm of wild type revealed only few cells undergoing PCD, enhanced PCD in *spr2* seeds may lead to or go along with delayed seed development. Although not checked by the TUNEL assay, the clearly aborted/shrunken endosperm in seeds of nontreated *jai1* plants (Fig. 2) points to an enhanced PCD in the nonstressed mutant. This fits into the hypothesis that seed-coat-generated OPDA as well as OPDA generated by wounding in *jai1* may attribute to the known up-regulation of stress-responsive genes such as those encoding heat shock proteins or glutathione-*S*-transferases, all involved in detoxification and specifically up-regulated by OPDA (Taki et al., 2005; Mueller et al., 2008). In light of these data, seed-coat-generated OPDA may attribute to detoxification of a tissue necessary for nutritional supply of the developing embryo.

Another scenario might result from the loss of defense gene expression of OPDA- and JA-induced genes, all affected in *jai1*, *35S*::*SlAOC-RNAi*, and *spr2*. In developing flowers and seeds of tomato, gene expression of JA-inducible defense proteins such as PIN2 and DEFENSIN2 is up-regulated (Li et al., 2004; Stotz et al., 2009). Wild-type flowers accumulate more than 200 μg/mL soluble PIN2 protein, which is the most abundant JA-inducible protein of tomato. In *jai1* flowers, however, PIN2 is undetectable (Li et al., 2001, 2004). A scenario of JA activity was suggested for flower development before anthesis: An increased JA level upon developmental *AOC* up-regulation in ovules (Hause et al., 2000) might be followed by *PIN2* expression as detected by in situ hybridization (Supplemental Fig. S2), which in turn may lead to the decreased herbivore infestation in flowers (Damle et al., 2005). A similar scenario, which may occur in seed development, could be an OPDA-induced DEFENSIN formation known to affect seed production and embryo development (Stotz et al., 2009). Such a dual function of defense proteins in defense and development was repeatedly observed. It is tempting to speculate that seed-coat-generated OPDA might be active by inducing DEFENSIN formation in the endosperm.

Another picture could be an OPDA-dependent alteration of carbon availability during seed formation. In tomato a JA-inducible cell wall invertase (*LIN5*) is preferentially expressed in gynoecia and was suggested to function in carbohydrate supply of this flower organ (Godt and Roitsch, 1997). Indeed, RNAi lines significantly silenced in *LIN5* expression showed decreased seed size, seed production per fruit and fruit yield, and decreased content of hexoses (Zanor et al., 2009). Interestingly, in *LIN5*-silenced lines the content of oxylipins measured as JA content was decreased down to about 20% of the wild type. Thus, it is tempting to speculate that JA biosynthesis is required spatially and temporally for proper generation of sugars that in turn are required for development of tomato embryos/fruits. Another essential factor might be proper amino acid levels that are remarkably decreased in transgenic JA-deficient tomato fruits (Kausch et al., 2012).

In tomato flowers the LIN5-dependent generation of hexoses (Zanor et al., 2009) is regulated posttranslationally by a LIN5-specific inhibitor protein INVINH1, which gene is coexpressed with *LIN5* within the gynoecia (Jin et al., 2009). Silencing of *INVINH1* allowed extra invertase activity that led to more hexoses being available for developing seeds and was followed by an increase in seed weight and size (Jin et al., 2009). Interestingly, such invertase inhibitors are down-regulated by OPDA (Taki et al., 2005). Consequently, a high level of OPDA as shown here for the developing seed coat may down-regulate inhibitor expression, thereby allowing LIN5 to generate hexoses for proper embryo development, whereas *spr2* seeds are unable to repress inhibitor expression due to OPDA deficiency.

The proposed role of OPDA in embryo development described here is an additional example similar to OPDA-

specific, but JA-independent gene expression (Taki et al., 2005; Mueller et al., 2008; Ribot et al., 2008; Böttcher and Pollmann, 2009), protein pattern (Dueckershoff et al., 2008), and other processes mediated preferentially by OPDA such as fertility of *P. patens* (Stumpe et al., 2010), phytochrome A signaling and shade avoidance (Robson et al., 2010), or hypocotyl growth inhibition at reduced V-ATPase activity (Brüx et al., 2008). More recently, genetic and biochemical evidence showed that inhibition of seed germination well known for a long time as a characteristic response upon JA/JA-Ile treatment is not caused by the applied compounds but is mediated by endogenous accumulation of OPDA during seed development in a COI1-independent manner (Dave et al., 2011). Such JA-independent changes are further supported by a recent proteome analysis of wounded Arabidopsis leaves. Here, about 95% of the wound-stimulated changes occurred in the absence of JA and included preferentially redox-controlled enzymes in glutathione synthesis and transporters (Gfeller et al., 2011). The increasing number of examples on JA-independent OPDA-specific responses addresses the question for future work on OPDA perception and OPDA signaling.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Treatments

Tomato (*Solanum lycopersicon*) cv MicroTom, cv Lukullus, cv Castlemart, the mutants *jai1* (in cv MicroTom), *spr2*, and *acx1a* (in cv Castlemart), and transgenic lines of cv MicroTom carrying a *35S::SlAOC-RNAi* construct (see below) or an *SlAOC::GUS* construct (Stenzel et al., 2008) were grown in greenhouse as described (Wasternack et al., 1998). The flowers were hand pollinated and seeds were prepared from the emerging fruits at different DAP. Wounding of *jai1* plants was performed by squeezing of fully developed leaves with tweezers every day in a time period of 5 d after onset of flowering.

Quantitative RT-PCR

Total RNA was prepared from 100 mg of isolated seeds (21 DAP) using the Plant RNeasy extraction kit (Qiagen, www.qiagen.com) with additional DNase digestion step (RNase-free DNase set; Qiagen). For real-time RT-PCR analyses, 1 μg of total RNA was converted into cDNA with M-MLV reverse transcriptase, RNase H minus, point mutant (Promega, www.promega.com) according to the manufacturer's protocol using oligo(dT)19 primer. cDNA was diluted to fixed quantities (15 ng per reaction of reverse-transcribed total RNA).

For semiquantitative detection of *SlACX1A* and *SlACX1B* transcripts a SYBR green-based PCR assay (Applied Biosystems, www.appliedbiosystems.com) was performed. The following primers and annealing temperatures were used: *ACX1A* forward 5′-CAA ATG CTG TAT CAC TGG TTG ATG-3′, *ACX1A* reverse 5′-CCA TCA TAA CGT CCA AGA ATT GAA-3′, 60°C; *ACX1B* forward 5′-GGG CCT TGT ATA CTC AGG TTT G-3′, *ACX1B* reverse 5′-TTT CGG GTA CAC AAA TGA ACA GCA-3′, 60°C. To normalize transcript levels for differences in the efficiency of cDNA synthesis, transcript levels of the constitutively expressed *SlTIP4l* were measured using the following primers and temperature: *TIP4l* forward 5′-ATG GAG TTT TTG AGT CTT CTG C-3′, *TIP4l* reverse 5′-GCT GCG TTT CTG GCT TAG G-3′, 60°C (Expósito-Rodríguez et al., 2008). The reactions were performed in a Mx 3000P QPCR system (Stratagene, www.stratagene.com) with the following protocol: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min, and 95°C for 1 min, and a subsequent standard dissociation protocol. As control for genomic DNA contamination, 15 ng of total nontranscribed RNA was used under the same conditions as described above. All assays were performed from three biological replicates and in three technical replicates each. Relative expression levels were calculated by the comparative Ct method including normalization to the constitutively expressed gene.

Extraction and Quantitative Analysis of Oxylipins

Seeds of at least five different fruits of at least three different plants were collected at different DAP. Seeds from 27 DAP were used for mechanical separation of seed coat tissues (integument and nucellus) and embryos using a stereo microscope. All materials (about 50–500 mg fresh weight) were immediately frozen in liquid nitrogen. Extraction, purification, and quantification of OPDA, dinor-OPDA, JA, 11-OH-JA, and 12-OH-JA were performed as described (Stenzel et al., 2003; Miersch et al., 2008).

Generation and Characterization of *35S::SlAOC-RNAi* Lines

For the *SlAOC*-repression constructs, an inverted-repeat construct was created using a *Bam*HI/*Xho*I fragment (423 bp) from the 3′ end of the *SlAOC*-cDNA. The inverted fragments were separated by a spacer containing 1,079 bp of the GUS gene. The RNAi construct was inserted into pBI101.1 (Clonetech, www.clonetech.com) between the cauliflower mosaic virus 35S promoter and the NOS terminator, checked by sequencing, and transformed into *Agrobacterium tumefaciens* strain GV3101. Transgenic tomato cv MicroTom were generated by *Agrobacterium*-mediated gene transfer essentially as described for generation of *SlAOC::GUS* lines (Stenzel et al., 2008). Analyses of integration numbers by Southern blot, level of *SlAOC* mRNA and protein were performed using primary transformants and T1 lines as described previously (Stenzel et al., 2003, 2008).

Histochemical Analyses

GUS activity analyses in intact seeds and isolated embryos were performed according to Stenzel et al. (2008). For analysis of seed morphology, developing seeds of wild-type (cv MicroTom) and *jai1* plants were fixed in 3% (v/v) sodium-cacodylate-buffered glutaraldehyde (pH 7.2), postfixed with 1% (w/v) OsO4 solution, dehydrated in an ethanol series, and embedded in epoxy resin (Spurr, 1969). Semithin sections (1-μm thickness) were stained with toluidine blue. For immunocytochemical analysis of SlAOC, in situ hybridization, and TUNEL assays, tissues were fixed with 4% (w/v) paraformaldehyde/0.1% (v/v) Triton X-100 in phosphate-buffered saline (PBS) immediately after harvest and embedded in polyethylene glycol 1500 as described (Isayenkov et al., 2005).

Immunodecoration and visualization of AOC protein was performed on 2-μm-thick sections with an antibody raised against recombinant SlAOC followed by an anti-rabbit IgG antibody either conjugated with AlexaFluor 488 (Invitrogen, www.invitrogen.com) or conjugated with alkaline phosphatase (Chemicon, www.chemicon.com) as described (Hause et al., 2000).

In situ hybridization at 5-μm-thick sections was carried out as outlined previously (Tretner et al., 2008) using digoxigenin-labeled antisense and sense RNA probes obtained from *PIN2* cDNA by in vitro transcription.

For TUNEL assay, 2-μm-thick sections were pretreated with 1 μg mL^{-1} proteinase K in 10 mM Tris-HCl (pH 8.0) at 37°C for 10 min. After washing with PBS, TUNEL reaction was carried out at 37°C for 60 min using the Fluorescein FragEL DNA fragmentation detection kit (Calbiochem, www.merck-chemicals.com). Positive controls were generated by pretreatment of sections with 1 μg mL^{-1} DNase I in PBS at 37°C for 15 min. After washing, sections were stained with 1 μg mL^{-1} DAPI (Sigma-Aldrich, www.sigmaaldrich.com) in PBS.

All sections were observed with an AxioImager (Zeiss, www.zeiss.de), pictures were taken with an Axiocam (Zeiss), and processed through Photoshop 8.0.1 (Adobe, www.adobe.com).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY817109.1 (ACX1A), AY817110.1 (ACX1B), X94946.1 (PIN2), and SGN-U321250 (TIP41).

Supplemental Data

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

Supplemental Figure S1. *SlAOC* mRNA and protein as well as OPDA and JA levels are decreased in wounded leaves of *35S::SlAOC-RNAi* lines compared to the wild-type MicroTom.

Supplemental Figure S2. The JA-responsive *PIN2* gene is preferentially expressed in ovules of unpollinated flowers and in developing embryos.

Supplemental Figure S3. Positive controls for TUNEL analysis in seeds of the wild-type Castlemart and the *spr2* mutant.

ACKNOWLEDGMENTS

We thank Birgit Ortel and Susann Gürtler for dependable technical assistance. Claudia Mönchmeier is acknowledged for preparing sections of *jai1* and wild-type seeds. We thank Gregg Howe for supply of *spr2*, *acx1a*, and *jai1* seeds and his continuous support and discussion of this work.

Received December 20, 2011; accepted February 10, 2012; published February 14, 2012.

LITERATURE CITED

Balbi V, Lomax TL (2003) Regulation of early tomato fruit development by the *diageotropica* gene. Plant Physiol **131:** 186–197

Berger F, Grini PE, Schnittger A (2006) Endosperm: an integrator of seed growth and development. Curr Opin Plant Biol **9:** 664–670

Boss WF, Sederoff HW, Im YJ, Moran N, Grunden AM, Perera IY (2010) Basal signaling regulates plant growth and development. Plant Physiol **154:** 439–443

Böttcher C, Pollmann S (2009) Plant oxylipins: plant responses to 12-oxo-phytodienoic acid are governed by its specific structural and functional properties. FEBS J **276:** 4693–4704

Browse J (2009a) Jasmonate passes muster: a receptor and targets for the defense hormone. Annu Rev Plant Biol **60:** 183–205

Browse J (2009b) The power of mutants for investigating jasmonate biosynthesis and signaling. Phytochemistry **70:** 1539–1546

Browse J, Howe GA (2008) New weapons and a rapid response against insect attack. Plant Physiol **146:** 832–838

Brüx A, Liu T-Y, Krebs M, Stierhof Y-D, Lohmann JU, Miersch O, Wasternack C, Schumacher K (2008) Reduced V-ATPase activity in the *trans*-Golgi network causes oxylipin-dependent hypocotyl growth inhibition in *Arabidopsis*. Plant Cell **20:** 1088–1100

Chen H, Jones AD, Howe GA (2006) Constitutive activation of the jasmonate signaling pathway enhances the production of secondary metabolites in tomato. FEBS Lett **580:** 2540–2546

Chung HS, Niu Y, Browse J, Howe GA (2009) Top hits in contemporary JAZ: an update on jasmonate signaling. Phytochemistry **70:** 1547–1559

Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonates in plants. Annu Rev Plant Physiol Plant Mol Biol **48:** 355–381

Damle MS, Giri AP, Sainani MN, Gupta VS (2005) Higher accumulation of proteinase inhibitors in flowers than leaves and fruits as a possible basis for differential feeding preference of *Helicoverpa armigera* on tomato (*Lycopersicon esculentum* Mill, Cv. Dhanashree). Phytochemistry **66:** 2659–2667

Dave A, Hernández ML, He Z, Andriotis VME, Vaistij FE, Larson TR, Graham IA (2011) 12-Oxo-phytodienoic acid accumulation during seed development represses seed germination in *Arabidopsis*. Plant Cell **23:** 583–599

Devoto A, Ellis C, Magusin A, Chang H-S, Chilcott C, Zhu T, Turner JG (2005) Expression profiling reveals *COI1* to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. Plant Mol Biol **58:** 497–513

Dueckershoff K, Mueller S, Mueller MJ, Reinders J (2008) Impact of cyclopentenone-oxylipins on the proteome of *Arabidopsis thaliana.* Biochim Biophys Acta **1784:** 1975–1985

Dumas C, Rogowsky P (2008) Fertilization and early seed formation. C R Biol **331:** 715–725

Expósito-Rodríguez M, Borges AA, Borges-Pérez A, Pérez JA (2008) Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol **8:** 131

Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C, Solano R (2009) (+)-7-*iso*-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol **5:** 344–350

Fowler JH, Narváez-Vásquez J, Aromdee DN, Pautot V, Holzer FM, Walling LL (2009) Leucine aminopeptidase regulates defense and wound signaling in tomato downstream of jasmonic acid. Plant Cell **21:** 1239–1251

Gfeller A, Baerenfaller K, Loscos J, Chételat A, Baginsky S, Farmer EE (2011) Jasmonate controls polypeptide patterning in undamaged tissue in wounded Arabidopsis leaves. Plant Physiol **156:** 1797–1807

Glauser G, Dubugnon L, Mousavi SAR, Rudaz S, Wolfender J-L, Farmer EE (2009) Velocity estimates for signal propagation leading to systemic jasmonic acid accumulation in wounded Arabidopsis. J Biol Chem **284:** 34506–34513

Godt DE, Roitsch T (1997) Regulation and tissue-specific distribution of mRNAs for three extracellular invertase isoenzymes of tomato suggests an important function in establishing and maintaining sink metabolism. Plant Physiol **115:** 273–282

Greenberg JT (1996) Programmed cell death: a way of life for plants. Proc Natl Acad Sci USA **93:** 12094–12097

Hause B, Stenzel I, Miersch O, Maucher H, Kramell R, Ziegler J, Wasternack C (2000) Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J **24:** 113–126

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annu Rev Plant Biol **59:** 41–66

Isayenkov S, Mrosk C, Stenzel I, Strack D, Hause B (2005) Suppression of allene oxide cyclase in hairy roots of *Medicago truncatula* reduces jasmonate levels and the degree of mycorrhization with *Glomus intraradices.* Plant Physiol **139:** 1401–1410

Ishiguro S, Kwai-Oda A, Ueda J, Nishida I, Okada K (2001) The *DEFECTIVE IN ANTHER DEHISCENCE1* gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation. Plant Cell **13:** 2191–2209

Jin Y, Ni D-A, Ruan Y-L (2009) Posttranslational elevation of cell wall invertase activity by silencing its inhibitor in tomato delays leaf senescence and increases seed weight and fruit hexose level. Plant Cell **21:** 2072–2089

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc Natl Acad Sci USA **105:** 7100–7105

Kausch K, Sobolev A, Goyal R, Fatima T, Laila-Beevi R, Saftner R, Handa A, Mattoo A (2012) Methyl jasmonate deficiency alters cellular metabolome, including the aminome of tomato (*Solanum lycopersicum* L.) fruit. Amino Acids **42:** 843–856

Koo AJK, Gao X, Jones AD, Howe GA (2009) A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J **59:** 974–986

Koo AJK, Howe GA (2009) The wound hormone jasmonate. Phytochemistry **70:** 1571–1580

Laudert D, Schaller F, Weiler EW (2000) Transgenic *Nicotiana tabacum* and *Arabidopsis thaliana* plants overexpressing allene oxide synthase. Planta **211:** 163–165

Lay FT, Brugliera F, Anderson MA (2003) Isolation and properties of floral defensins from ornamental tobacco and petunia. Plant Physiol **131:** 1283–1293

Li C, Liu G, Xu C, Lee GI, Bauer P, Ling H-Q, Ganal MW, Howe GA (2003) The tomato suppressor of prosystemin-mediated responses2 gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression. Plant Cell **15:** 1646–1661

Li C, Schilmiller AL, Liu G, Lee GI, Jayanty S, Sageman C, Vrebalov J, Giovannoni JJ, Yagi K, Kobayashi Y, et al (2005) Role of beta-oxidation in jasmonate biosynthesis and systemic wound signaling in tomato. Plant Cell **17:** 971–986

Li L, Li C, Howe GA (2001) Genetic analysis of wound signaling in tomato: evidence for a dual role of jasmonic acid in defense and female fertility. Plant Physiol **127:** 1414–1417

Li L, Li C, Lee GI, Howe GA (2002) Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. Proc Natl Acad Sci USA **99:** 6416–6421

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe GA (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16:** 126–143

McConn M, Browse J (1996) The critical requirement for linolenic acid is pollen development, not photosynthesis, in an *Arabidopsis* mutant. Plant Cell **8:** 403–416

Miersch O, Knöfel H-D, Schmidt J, Kramell R, Parthier B (1998) A jasmonic acid conjugate, *N*[(-)-jasmonoyl]-tyramine, from *Petunia* pollen. Phytochemistry **47:** 327–329

Miersch O, Neumerkel J, Dippe M, Stenzel I, Wasternack C (2008) Hydroxylated jasmonates are commonly occurring metabolites of jasmonic acid and

contribute to a partial switch-off in jasmonate signaling. New Phytol **177:** 114–127

Miersch O, Weichert H, Stenzel I, Hause B, Maucher H, Feussner I, Wasternack C (2004) Constitutive overexpression of allene oxide cyclase in tomato (*Lycopersicon esculentum* cv. Lukullus) elevates levels of some jasmonates and octadecanoids in flower organs but not in leaves. Phytochemistry **65:** 847–856

Milligan SB, Gasser CS (1995) Nature and regulation of pistil-expressed genes in tomato. Plant Mol Biol **28:** 691–711

Mueller S, Hilbert B, Dueckershoff K, Roitsch T, Krischke M, Mueller MJ, Berger S (2008) General detoxification and stress responses are mediated by oxidized lipids through TGA transcription factors in *Arabidopsis*. Plant Cell **20:** 768–785

Patton DA, Schetter AL, Franzmann LH, Nelson K, Ward ER, Meinke DW (1998) An embryo-defective mutant of Arabidopsis disrupted in the final step of biotin synthesis. Plant Physiol **116:** 935–946

Pena-Cortes H, Willmitzer L, Sanchez-Serrano JJ (1991) Abscisic acid mediates wound induction but not developmental-specific expression of the proteinase inhibitor II gene family. Plant Cell **3:** 963–972

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol **5:** 308–316

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell **12:** 707–720

Ribot C, Zimmerli C, Farmer EE, Reymond P, Poirier Y (2008) Induction of the Arabidopsis *PHO1;H10* gene by 12-oxo-phytodienoic acid but not jasmonic acid via a *CORONATINE INSENSITIVE1*-dependent pathway. Plant Physiol **147:** 696–706

Robson F, Okamoto H, Patrick E, Harris S-R, Wasternack C, Brearley C, Turner JG (2010) Jasmonate and phytochrome A signaling in *Arabidopsis* wound and shade responses are integrated through JAZ1 stability. Plant Cell **22:** 1143–1160

Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, Goldberg RB (2000) The *Arabidopsis DELAYED DEHISCENCE1* gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell **12:** 1041–1061

Schaller A, Stintzi A (2009) Enzymes in jasmonate biosynthesis—structure, function, regulation. Phytochemistry **70:** 1532–1538

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu F-F, Sharon M, Browse J, et al (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature **468:** 400–405

Spurr AR (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res **26:** 31–43

Staswick PE, Tiryaki I (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. Plant Cell **16:** 2117–2127

Stenzel I, Hause B, Maucher H, Pitzschke A, Miersch O, Ziegler J, Ryan CA, Wasternack C (2003) Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato—amplification in wound signalling. Plant J **33:** 577–589

Stenzel I, Hause B, Proels R, Miersch O, Oka M, Roitsch T, Wasternack C (2008) The AOC promoter of tomato is regulated by developmental and environmental stimuli. Phytochemistry **69:** 1859–1869

Stintzi A, Browse J (2000) The Arabidopsis male-sterile mutant, *opr3*, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci USA **97:** 10625–10630

Stotz HU, Spence B, Wang Y (2009) A defensin from tomato with dual function in defense and development. Plant Mol Biol **71:** 131–143

Stumpe M, Göbel C, Faltin B, Beike AK, Hause B, Himmelsbach K, Bode J, Kramell R, Wasternack C, Frank W, et al (2010) The moss *Physcomitrella patens* contains cyclopentenones but no jasmonates: mutations in allene oxide cyclase lead to reduced fertility and altered sporophyte morphology. New Phytol **188:** 740–749

Taki N, Sasaki-Sekimoto Y, Obayashi T, Kikuta A, Kobayashi K, Ainai T, Yagi K, Sakurai N, Suzuki H, Masuda T, et al (2005) 12-Oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in Arabidopsis. Plant Physiol **139:** 1268–1283

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448:** 661–665

Tretner C, Huth U, Hause B (2008) Mechanostimulation of *Medicago truncatula* leads to enhanced levels of jasmonic acid. J Exp Bot **59:** 2847–2856

Wang H, Schauer N, Usadel B, Frasse P, Zouine M, Hernould M, Latché A, Pech J-C, Fernie AR, Bouzayen M (2009) Regulatory features underlying pollination-dependent and -independent tomato fruit set revealed by transcript and primary metabolite profiling. Plant Cell **21:** 1428–1452

Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot (Lond) **100:** 681–697

Wasternack C, Hause B (2002) Jasmonates and octadecanoids: signals in plant stress response and development. Progr Nucl Acid Res Mol Biol **72:** 165–221

Wasternack C, Ortel B, Miersch O, Kramell R, Beale M, Greulich F, Feussner I, Hause B, Krumm T, Boland W, et al (1998) Diversity in octadecanoid-induced gene expression of tomato. J Plant Physiol **152:** 345–352

Xie D-X, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280:** 1091–1094

Yan J, Zhang C, Gu M, Bai Z, Zhang W, Qi T, Cheng Z, Peng W, Luo H, Nan F, et al (2009) The *Arabidopsis* CORONATINE INSENSITIVE1 protein is a jasmonate receptor. Plant Cell **21:** 2220–2236

Zanor MI, Osorio S, Nunes-Nesi A, Carrari F, Lohse M, Usadel B, Kühn C, Bleiss W, Giavalisco P, Willmitzer L, et al (2009) RNA interference of LIN5 in tomato confirms its role in controlling Brix content, uncovers the influence of sugars on the levels of fruit hormones, and demonstrates the importance of sucrose cleavage for normal fruit development and fertility. Plant Physiol **150:** 1204–1218

Ziegler J, Stenzel I, Hause B, Maucher H, Hamberg M, Grimm R, Ganal M, Wasternack C (2000) Molecular cloning of allene oxide cyclase: the enzyme establishing the stereochemistry of octadecanoids and jasmonates. J Biol Chem **275:** 19132–19138