Jasmonic Acid Levels Are Reduced in COMATOSE ATP-Binding Cassette Transporter Mutants. Implications for Transport of Jasmonate Precursors into Peroxisomes[1]

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We provide evidence that the peroxisomal ATP-binding cassette (ABC) transporter COMATOSE (CTS) is involved in the biosynthesis of jasmonic acid (JA) in Arabidopsis (*Arabidopsis thaliana*) leaves. Basal JA levels were greatly reduced but not completely abolished in two *cts* mutant alleles, and JA production in response to wounding showed slower kinetics and reached a lower level than wild type. We propose the operation of two parallel pathways for peroxisomal import of jasmonate precursors, a route that requires CTS function and a parallel leak pathway involving anion trapping.

It is well established that the bioactive, lipid-derived compounds collectively known as jasmonates play important roles in plant defense, metabolism, and development (Farmer and Ryan, 1992; Creelman and Mulpuri, 2002). A biosynthetic pathway for JA was proposed in 1983 by Vick and Zimmerman (1983), in which JA is formed from the unsaturated fatty acid, linolenic acid (18:3), an abundant octadecanoid in higher plant membranes. In Arabidopsis, a parallel, hexadecanoid pathway based on the 16:3 fatty acid is also present (Weber et al., 1997). Most of the enzymes involved in the 18:3 pathway have now been identified by a combination of classic biochemical and genetic approaches (Creelman and Mulpuri, 2002; Turner et al., 2002). Following phospholipase-dependent release of 18:3 from chloroplast membrane lipids, molecular oxygen is introduced by 13-lipoxygenase activity to generate 13-hydroperoxy octadecatrienoic acid. Allene oxide synthase catalyzes the formation of an unstable intermediate, 12,13-epoxy-octadecatrienoic acid, which is converted to (9*S*,13*S*)-12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase. The synthesis of OPDA occurs in the chloroplast, and in Arabidopsis more than 90% of OPDA is esterified to chloroplast galactolipids (Stelmach et al., 2001). In subsequent steps, OPDA is reduced to 3-oxo-2-(2′-pentenyl)-cyclopentane-1-octanoic acid (OPC:8), which is then shortened by three rounds of peroxisomal β-oxidation to yield JA. The involvement of specific isoforms of acyl-CoA oxidase (ACX1) and thiolase (KAT2) in the β-oxidation steps of wound-induced leaf JA biosynthesis has recently been demonstrated (Cruz Castillo et al., 2004). Thus, JA biosynthesis is initiated in the chloroplast and terminates in the peroxisome. Although Arabidopsis has at least three isoforms of 12-oxo-phytodienoate reductase, only OPR3 reduces the 9*S*,13*S*-stereoisomer of OPDA, the biological precursor of JA (Schaller et al., 2000). Since OPR3 is located in the peroxisome (Strassner et al., 2002), it is clear that OPDA or other intermediates in JA synthesis must be transported from the chloroplast to the peroxisome, but the nature of these transport steps is unknown. It has generally been assumed that OPDA is freely membrane permeable because of its structural similarity to fatty acids, but since OPDA is an important signaling molecule in its own right (Blechert et al., 1999; Stintzi et al., 2001), it is plausible that control of its movement between subcellular compartments would be highly regulated (Stenzel et al., 2003). This could implicate membrane-bound transporters in the chloroplast and/or peroxisome.

A role in regulating fatty acid or fatty acyl-CoA import into the peroxisome has recently been demonstrated for the CTS ABC transporter (Footitt et al., 2002), also known as PXA1 and PED3 (Zolman et al., 2001; Hayashi et al., 2002). *cts* mutant seeds retain oil bodies, accumulate very long-chain fatty acyl-CoAs, and do not germinate in the absence of classical seed dormancy-breaking treatments (Footitt et al., 2002).

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Moreover, *cts* mutant seedlings cannot establish in the absence of Suc. Interestingly, alleles of *cts* were identified independently in screens for resistance to indole butyric acid (IBA) and the synthetic auxin precursor 2,4-dichlorophenoxy butyric acid (2,4-DB; Zolman et al., 2001; Hayashi et al., 2002). In wild-type Arabidopsis, these compounds are converted to bioactive auxins by β-oxidation. Mutation of CTS blocks β-oxidation of IBA and 2,4-DB, suggesting that CTS is also able to transport (or regulate the transport of) these compounds. This implies that the substrate specificity of CTS is quite broad. Many, but not all, ABC transporters are able to accept a variety of structurally quite diverse compounds (Theodoulou, 2000). Common features of the potential CTS substrates are an acyl chain of at least four carbons, terminating in a carboxyl group that is potentially esterified to CoA (Fig. 1). Examination of the structure of IBA and 2,4-DB suggests that a ring structure also is accommodated by the transporter. Given the structural similarities of these molecules to OPDA, which is derived from linolenic acid (Fig. 1), we hypothesized that CTS might also mediate the transport of this molecule and thus participate in JA biosynthesis.

Levels of JA and oxylipin intermediates were measured in two *cts* mutant alleles and their respective wild types by liquid chromatography-mass spectrometry (Fig. 2). The basal level of JA was around 30 ng g^{-1} fresh weight (FW) in wild-type plants. *cts* mutant alleles contained considerably less JA than wild type: 4.084 $\pm$ 2.346 and 1.236 $\pm$ 0.885 ng g^{-1} FW in *cts1* and *cts2*, respectively. OPDA levels were similar in *cts1* and Landsberg *erecta* (L*er*), which might reflect feedback inhibition of the pathway, as is the case for OPR3 (Mussig et al., 2000). In agreement with this, unwounded *opr3* leaves were found to contain less free OPDA than expected by the block in OPR3 (Stenzel et al., 2003). However, OPDA was increased significantly in *cts2* relative to the wild type, Wassilewskija (Ws). Since both *cts1* and *cts2* are effectively null alleles, this suggests possible ecotype-specific differences in the regulation of the JA pathway that are not related to CTS. Interestingly, in *cts2*, the levels of 13-hydroxy-12-oxo-octadecadienoic acid (α-ketol) and 9-hydroxy-12-oxo-octadecadienoic acid (γ-ketol) were elevated relative to the wild type. An increase in these compounds, which are formed by nonenzymic hydrolysis of unstable allene oxides (Grechkin et al., 1991), is indicative of an increased ability to oxidize 18:3 (Weber et al., 1997). In a previous report, α-ketol formation increased in response to dnOPDA treatment of Arabidopsis (Weber et al., 1997), suggesting that the increase in ketols in *cts2* might reflect the elevated levels of OPDA in this mutant.

Analysis of the Arabidopsis genome indicates that CTS is a single gene (Sánchez-Fernández et al., 2001) and is therefore likely to participate in β-oxidation throughout the plant. In accordance with this, CTS is expressed in all tissues (http://www.cbs.umn.edu/arabidopsis). Quantitative reverse transcription-PCR, shown in Figure 3A, demonstrated that floral tissues contain about 2-fold more CTS transcript than siliques

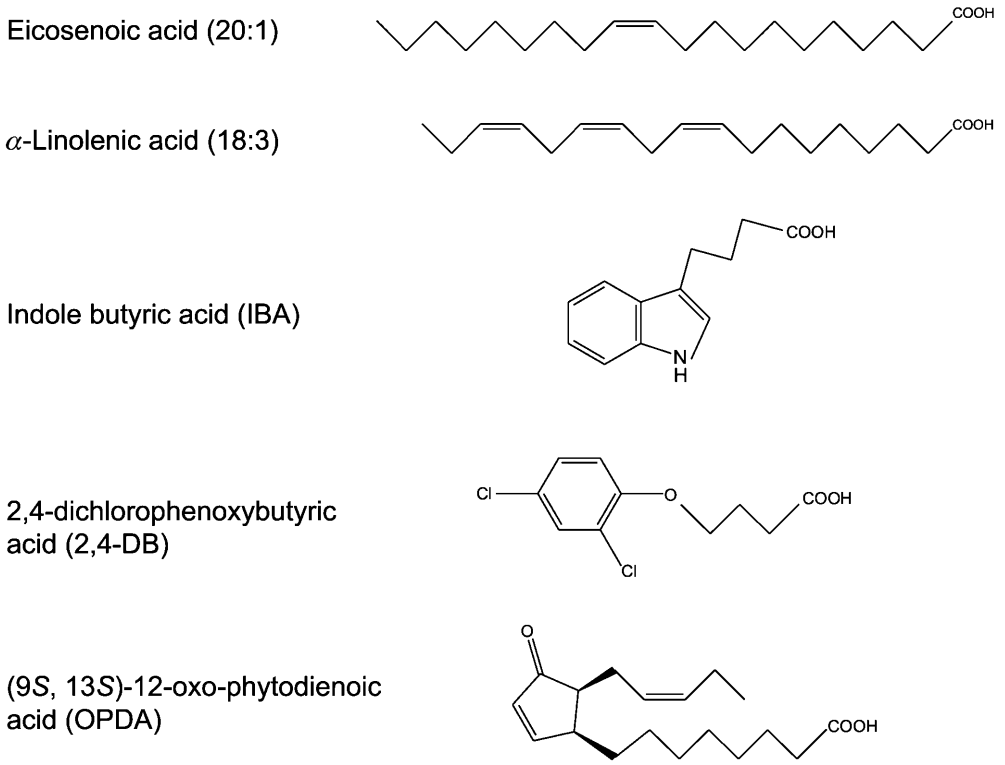

Figure 1. Chemical structures of potential CTS substrates. Free acids are shown, but their CoA esters are also possible substrates.

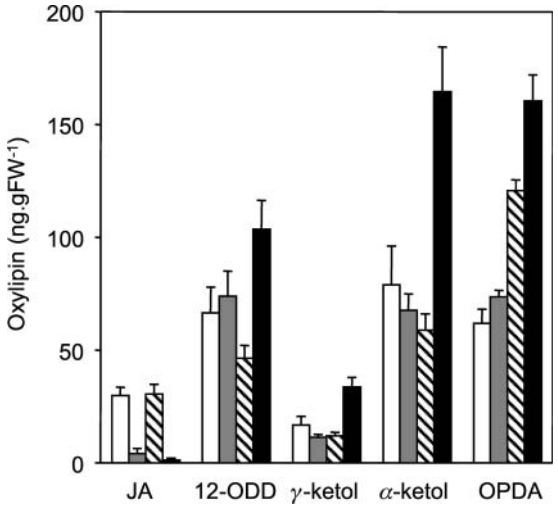

Figure 2. *cts* mutants are JA deficient. Measurement of JA and oxylipin intermediates in *cts* mutants and respective wild types is shown. White bars, Wild-type L*er*; gray bars, *cts1*; hatched bars, wild-type Ws; black bars, *cts2*. Values are means ± SE from eight independent samples, each of which was analyzed in duplicate. Surface-sterilized seeds of the mutants *cts1* and *cts2* were plated on Gamborg's B5 salts, 0.7% (w/v) agarose, pH 5.8, for 4 d at 22°C in continuous light (150 μmol m^{-2} s^{-1}) to confirm the phenotype. They were then transferred to plates, as above, supplemented with 0.5% (w/v) Suc and nicked to allow germination. Both mutants and wild-type seeds were stratified on the same media in the dark for 2 d at 4°C followed by germination for 7 d at 22°C. Seedlings were then transferred to soil in cellular trays and grown for 13 d under a long-day cycle (22°C, 16 h light/18°C, 8 h dark) at 150 to 170 μmol m^{-2} s^{-1}, 60% relative humidity. For each mutant and wild type, there were 8 trays of 20 seedlings; from each tray, two replicate samples of rosette leaf were harvested, weighed, and immediately frozen in liquid nitrogen. Oxylipins were quantified from 200 mg of tissue that was ground to powder and extracted overnight in 70:30 (v/v) acetone:50 mM citric acid with 20 ng prostaglandin A1 (Sigma, St. Louis) added as an internal standard. Oxylipins were extracted from the aqueous phase by partitioning 3 times with diethyl ether, evaporating to dryness, and resuspending in 60% methanol prior to HPLC tandem mass spectrometry analysis. Separation was achieved using a LUNA C18(2) 150 × 2-mm column (Phenomenex, Torrance, CA) and a methanol-water-0.2% formic acid gradient. Eluted compounds were fragmented by targeted tandem mass spectrometry using an LCQ mass spectrometer (Thermo Separation Products, Riviera Beach, FL), and the daughter ions identified and quantified using response factors calibrated between prostaglandin A1 and authentic oxylipin standards (Larodan, Malmo, Sweden).

and 4- to 5-fold more than roots or rosette leaves. In order to relate these findings to a potential role in JA biosynthesis, the expression of a JA-responsive gene was examined in the *cts1* mutant. Arabidopsis vegetative storage protein 1 (VSP1) is expressed preferentially in flowers and buds and only at very low levels in other tissues (Utsugi et al., 1996, 1998). VSP1 expression is induced in vegetative tissue by methyl JA and coronatine in a COI1-dependent manner (Benedetti et al., 1995) and is also induced by wounding (Berger et al., 1995, Utsugi et al., 1998). Moreover, VSP1 is constitutively expressed in the *cev1* mutant that has constitutively active jasmonate- and ethylene-signaling pathways (Ellis and Turner, 2001). Thus, VSP1 was selected as a well-characterized JA-responsive gene whose expression might be detected in unwounded or untreated tissue. Primers specific for VSP1 (At5g24780) were designed and the levels of transcript measured by quantitative PCR in samples grown under identical conditions to those used for the experiment shown in Figure 2. In agreement with

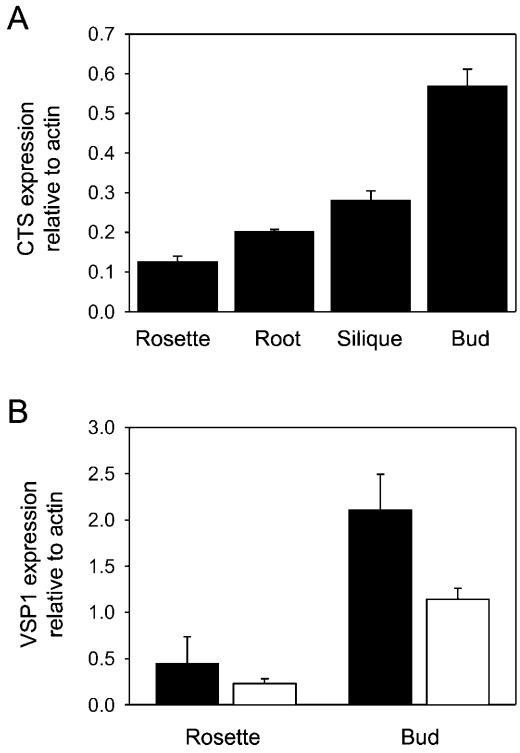

Figure 3. Transcript levels of CTS and VSP in tissues measured by quantitative real-time PCR. A, Expression of CTS relative to actin in wild-type L*er* plants. B, Expression of VSP1 relative to actin in wild-type L*er* (black bars) and *cts1* mutant plants (white bars). Plants were grown under the same conditions as the samples for basal JA analysis (Fig. 2). Tissues were harvested, weighed, and frozen immediately in liquid nitrogen. RNA was extracted from 100-mg tissue using an RNeasy mini kit and treated with RNase-free DNase I (Qiagen, Westburg, The Netherlands). Total RNA (3 μg) was reverse transcribed with Superscript II reverse transcriptase (Invitrogen Life Technologies, Paisley, UK) using a mixture of oligo$(dT)_{15}$ and random hexamer primers. PCR reactions were carried out in triplicate on three independent RNA samples for each tissue, using the 300-nmol primers QCTSF1 (5′-GAGATTAGGCATGGCACGTT-3′) and QCTSR1 (5′-GTCGCATTTGTGCATTCATC-3′) or VSP1right (5′-GGCACCGTGTCGAAGTTTAT-3′) and VSP1left (5′-CTCTTGGTCGCTACGGTCTC-3′). Identical reactions were carried out using the 300-nmol primers ACT2F (5′-CTCAGGTATCGCTGACCGTA-3′) and ACT2R (5′-GAGATCCACATCTGCTGGAAT-3′). PCR cycling conditions were 95°C, 3 min, followed by 40 cycles of 95°C, 15 s; 60°C, 30 s. Data were analyzed using the Icycler instrument software (Bio-Rad, Hemel Hempstead, UK), which produced cycle threshold values and relative PCR product levels calculated from the standard curve. Values obtained for CTS or VSP1 were divided by those obtained from actin in the same sample to correct for any variation in the amount of input cDNA and are presented as expression relative to actin.

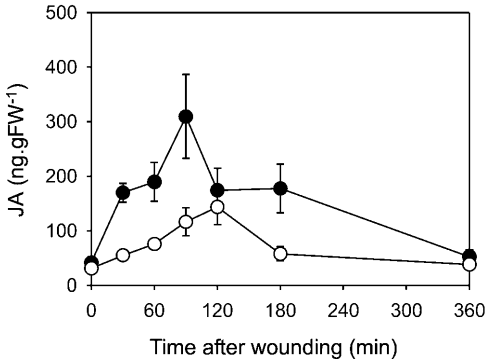

Figure 4. Time course of JA accumulation in *cts2* mutant and wild type following wounding of leaves. Arabidopsis plants were grown and wounded as described by Weber et al. (1997). Briefly, plants were grown under short-day conditions (9 h light; 150 μmol m^{-2} s^{-1} at 23°C, 70% relative humidity). The apical halves of rosette leaves from 4-week-old plants were wounded with forceps and kept under light (150 μmol m^{-2} s^{-1}) for up to 3 h. JA was quantified as described in the legend to Figure 2. White symbols, *cts2*; black symbols, Ws. Values are means ± SE of five independent determinations.

previous reports, VSP1 was expressed at low levels in vegetative tissue but was highly expressed in floral tissue, and the level was decreased by almost one-half in flowers of the *cts1* mutant (Fig. 3B). As the floral expression of VSP1 is known to be COI-dependent (Benedetti et al., 1995), this is consistent with the fact that *cts* mutants exhibit reduced JA.

The induction of JA biosynthesis by wounding is well studied, and it is becoming evident that developmentally regulated JA biosynthesis and wound-induced JA biosynthesis occur via distinct but overlapping pathways (Turner et al., 2002). We therefore examined the role of CTS in wound-induced JA formation. In this experiment, basal JA levels were not significantly different in Ws and *cts2* (41.404 ± 11.000 and 31.065 ± 8.636 ng g^{-1} FW, respectively). This may reflect the fact that plants were grown under short days, compared to the long-day growth conditions, in the experiment reported in Figure 2, and that the plants were of a different developmental age. However, a marked difference was seen following wounding: In wild-type leaves, wounding induced an increase in JA that peaked 90 min after treatment at approximately 310 ng g^{-1} FW (Fig. 4). The kinetics of this response are consistent with published reports (McConn et al., 1997; Weber et al., 1997; Stenzel et al., 2003; Cruz Castillo et al., 2004). Although JA increased in response to wounding of *cts2* leaves, the response was slower, with JA peaking 2 h after wounding at a lower level of 144 ng g^{-1} FW.

Taken together, these data suggest a possible role for CTS in the peroxisomal import of JA precursors. The simplest interpretation of this finding is that CTS mediates ATP-dependent transport of OPDA or OPDA-CoA into the peroxisome. However, until CTS has been reconstituted for transport studies, other hypotheses cannot be ruled out; for example, CTS may regulate the activity of another transport system since dual roles as primary transporters and transport regulators have been established for a range of different ABC proteins (Higgins, 1995). It is also not clear whether free OPDA or a CoA-esterified form is transported into the peroxisome. Cytosolic addition of a large, polar CoA moiety to OPDA would render it membrane impermeable in the absence of a transporter. While Arabidopsis contains a large family of acyl-CoA synthetases (ACS) potentially capable of forming OPDA-CoA esters (Shockey et al., 2002, 2003), OPR3 accepts free OPDA as a substrate in vitro, which is consistent with the possibility that activation occurs following this reduction step in the peroxisome. It was concluded that the peroxisomal very long-chain ACSs LACS6 and LACS7 do not participate in jasmonate biosynthesis since the *lacs6/lacs7* double knockout is not male sterile (Fulda et al., 2004). However, the CTS-dependent pathway is intact in this double mutant. A *cts/lacs6/lacs7* triple mutant would resolve this issue. Alternatively, other members of the ACS family might catalyze the activation of OPDA or OPC:8.

It is evident from the data in Figures 2 to 4 that CTS contributes both to basal and wound-induced JA biosynthesis in leaves and that, potentially, it can fulfill this role in other tissues. However, since *cts* mutants contain low but measurable JA levels, CTS is not absolutely required for JA synthesis. Therefore, there

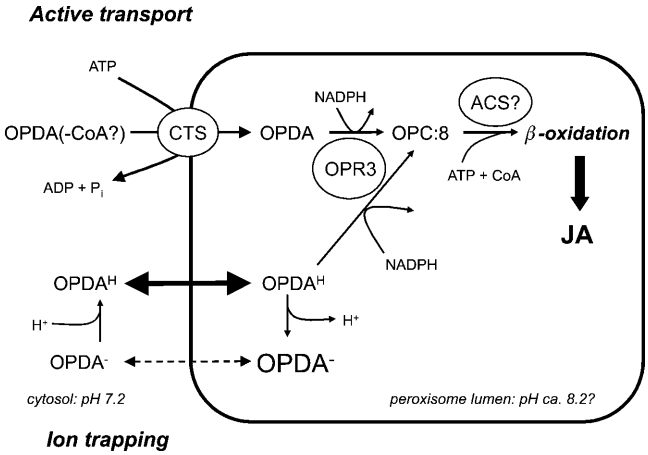

Figure 5. Model showing parallel pathways for import of jasmonate precursors into peroxisomes. In the CTS-dependent pathway, OPDA or its CoA ester is transported into the peroxisome in an ATP-dependent manner (active transport). It is possible that OPDA-CoA might be cleaved by peroxisomal thioesterase prior to reduction (Tilton et al., 2004; data not shown). Alternatively, OPDA-free acid ($OPDA^H$) is membrane permeable and can enter the peroxisome without the need for a transport protein. The peroxisome lumen is predicted to be more basic than the cytosol; therefore, a higher proportion of OPDA exists in the dissociated form ($OPDA^-$), which has much lower membrane permeability, resulting in ion trapping. In this pathway, the partitioning of OPDA between cytosol and peroxisome depends on the pH gradient between the two compartments. Inside the peroxisome, OPDA is reduced to OPC:8 by the action of OPR3, using NADPH as a cofactor. By analogy with fatty acids, OPC:8 would require CoA esterification prior to β-oxidation. The ACSs responsible for this hypothetical reaction are currently unknown.

must be an alternative route for uptake of OPDA into the peroxisome, either an unidentified transporter or a passive mechanism. Two physicochemical parameters largely define the membrane transport properties of weak acids such as OPDA: lipophilicity, as measured by the octanol-water partition coefficient (log K_{ow}), and acid strength, expressed as the pKa. Although values for OPDA have not been published, Dathe et al. (1993) demonstrated that JA is a weak acid with a pKa of 4.5 and a log K_{ow} of 1.76. Thus, depending on the proton concentration, JA exists in two forms, the lipophilic free acid and the essentially membrane-impermeable anion. In the absence of specific transport proteins, these physicochemical parameters predict that JA distributes according to the ion trap concept, leading to accumulation in alkaline compartments and depletion in acidic compartments. Peroxisomal pH measurements have not been published for plants, but use of targeted pH reporters indicated a pH of 8.2 in mammalian peroxisomes (Dansen et al., 2000) and the basic pI of plant peroxisomal lumen proteins suggests that plant peroxisomes are also likely to be basic compartments. Since cytosolic pH is in the range of 7.2 to 7.4 in plants (Kurkdjian and Guern, 1989), weak acids are predicted to be anion trapped in peroxisomes. The structure of OPDA suggests that it will have a very similar pKa to JA, but the longer acyl chain dictates that it is significantly more lipophilic, with an estimated log K_{ow} of 4.26 for the uncharged form (addition of 0.5 log K_{ow} unit for each extra CH_2 group). Thus, OPDA is likely to be ion trapped in peroxisomes but with relatively low efficiency, since the anionic form is sufficiently lipophilic to cross membranes, albeit to a lesser extent than the free acid. Subsequent reduction to OPC:8 would increase the chemical gradient and help to drive transport into the peroxisome. This passive mechanism could constitute a leak pathway for OPDA import, permitting JA biosynthesis in the absence of CTS (Fig. 5). This would explain the low basal level of JA in *cts* mutant alleles. The relative contribution of the two import pathways would be predicted to differ at different developmental stages and in response to different stimuli, depending not only on the level of CTS transport activity but also on the prevailing pH gradient between the cytosol and the peroxisome lumen. The response to wounding in *cts2*, in which basal JA is unchanged but where induction of JA synthesis is delayed and of reduced magnitude, is thus also consistent with anion trapping of a jasmonate precursor.

What are the other physiological consequences of compromised JA synthesis in *cts* mutants? Typically, Arabidopsis mutants defective in JA biosynthesis or perception are male sterile (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001). *cts* mutants, notably, are not male sterile. We propose that sufficient JA for male fertility is produced via the leak pathway in *cts* mutants. In agreement with this, McConn and Browse (1996) demonstrated that the threshold level of the JA precursor 18:3 for male fertility was less than 5% of total fatty acids (compared with 44% in wild type), strongly suggesting that JA can be reduced without resulting in male sterility. Previous work has shown that CTS is required for germination and seedling establishment (Footitt et al., 2002). The consequence of the involvement of CTS in JA biosynthesis as well as the established role in fatty acid transport suggest a metabolic and signaling role for CTS. The requirement for CTS in germination and seedling establishment can be interpreted in this light. The requirement for classic dormancy-breaking treatments to induce germination in *cts* suggests an involvement for CTS in endogenous signaling at the initiation of germination, whereas the requirement for CTS in seedling establishment represents the expression of the metabolic function of CTS. Involvement of CTS in the biosynthesis of JA suggests that CTS may have more subtle or as yet undefined functions during plant growth and begs the question of other substrates of CTS.

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