The COMATOSE ATP-Binding Cassette Transporter Is Required for Full Fertility in Arabidopsis[1][W][OA]

Steven Footitt[2], Daniela Dietrich, Aaron Fait, Alisdair R. Fernie, Michael J. Holdsworth, Alison Baker, and Frederica L. Theodoulou*

Crop Performance and Improvement Division, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, United Kingdom (S.F., D.D., F.L.T.); Division of Agricultural and Environmental Sciences, University of Nottingham, Loughborough, Leicestershire LE12 5RD, United Kingdom (D.D., M.J.H.); Max Planck Institute of Molecular Plant Physiology, 14476 Golm, Germany (A.F., A.R.F.); and Centre for Plant Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom (A.B.)

COMATOSE (*CTS*) encodes a peroxisomal ATP-binding cassette transporter required not only for β-oxidation of storage lipids during germination and establishment, but also for biosynthesis of jasmonic acid and conversion of indole butyric acid to indole acetic acid. *cts* mutants exhibited reduced fertilization, which was rescued by genetic complementation, but not by exogenous application of jasmonic acid or indole acetic acid. Reduced fertilization was also observed in thiolase (*kat2-1*) and peroxisomal acyl-Coenzyme A synthetase mutants (*lacs6-1*,*lacs7-1*), indicating a general role for β-oxidation in fertility. Genetic analysis revealed reduced male transmission of *cts* alleles and both *cts* pollen germination and tube growth in vitro were impaired in the absence of an exogenous carbon source. Aniline blue staining of pollinated pistils demonstrated that pollen tube growth was affected only when both parents bore the *cts* mutation, indicating that expression of *CTS* in either male or female tissues was sufficient to support pollen tube growth in vivo. Accordingly, abundant peroxisomes were detected in a range of maternal tissues. Although γ-aminobutyric acid levels were reduced in flowers of *cts* mutants, they were unchanged in *kat2-1*, suggesting that alterations in γ-aminobutyric acid catabolism do not contribute to the reduced fertility phenotype through altered pollen tube targeting. Taken together, our data support an important role for β-oxidation in fertility in Arabidopsis (*Arabidopsis thaliana*) and suggest that this pathway could play a role in the mobilization of lipids in both pollen and female tissues.

In oilseed plants, fatty acids are stored in the seed as triacylglycerol (TAG), which is metabolized by lipase activity and peroxisomal β-oxidation to yield acetyl-CoA. Subsequent conversion of acetyl-CoA to succinate via the glyoxylate cycle provides energy and carbon skeletons, which are essential for seedling development before the capacity for photosynthesis is established (Baker et al., 2006). In each turn of the β-oxidation spiral, fatty acid chains are shortened by two carbon units with the concomitant generation of acetyl-CoA. Core reactions of the pathway are catalyzed by three enzymes: acyl-CoA oxidase (ACX), multifunctional protein (MFP), and 3-ketoacyl-CoA thiolase (KAT), each of which is encoded by more than one gene in Arabidopsis (*Arabidopsis thaliana*; for review, see Graham and Eastmond, 2002; Baker et al., 2006). Prior to β-oxidation, substrates must be activated by esterification to CoA and imported into the peroxisome. Because Arabidopsis contains a large family of acyl-activating enzymes, only a subset of which are located in the peroxisome, it is likely that both free acids and CoA esters could be transported across the peroxisomal membrane (Shockey et al., 2002, 2003; Fulda et al., 2004; Theodoulou et al., 2006).

COMATOSE (*CTS*; also known as *PEROXISOME DEFECTIVE3* [*PED3*] and *A. thaliana PEROXISOMAL ABC TRANSPORTER1* [*AtPXA1*]) encodes a peroxisomal ATP-binding cassette transporter, which was identified in a genetic screen for positive regulators of germination (Russell et al., 2000). *cts-1* mutant seeds cannot germinate in the absence of classical dormancy-breaking treatments and do not establish in the absence of an exogenous energy source because of their inability to mobilize storage lipids by β-oxidation (Footitt et al., 2002, 2006). Mutant *cts* alleles have also been identified in screens for seedlings resistant to 2,4-dichlorophenoxybutyric acid and indole butyric acid (IBA; Zolman et al., 2001; Hayashi et al., 2002). These compounds are converted by one round of β-oxidation to the bioactive auxins, 2,4-dichlorophenoxyacetic acid and indole acetic acid (IAA), respectively,

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[2] Present address: Warwick HRI, Warwick University, Wellesbourne, Warwick CV35 9EF, UK.

* Corresponding author; e-mail freddie.theodoulou@bbsrc.ac.uk; fax 44–1582–763010.

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which stunt roots. These findings suggest that CTS either imports or regulates the peroxisomal import of a relatively wide range of substrates for β-oxidation.

We have shown recently that *CTS* also contributes to the synthesis of jasmonic acid (JA; Theodoulou et al., 2005). JA synthesis begins in the chloroplast, where membrane-derived linolenic acid (18:3) is converted to 12-oxophytodienoic acid (OPDA). OPDA exits the chloroplast and is imported into the peroxisome by a process requiring CTS, where it is reduced and then converted to JA by three cycles of β-oxidation (Schaller et al., 2004). JA levels are reduced, but not abolished, in *cts* mutants, indicating the existence of an additional, probably passive, route for uptake of JA precursors into the peroxisome (Theodoulou et al., 2005). Other authors have also demonstrated reduced JA in antisense lines or mutants in which β-oxidation is impaired; for example, plants deficient in *KAT2*, *ACX1*, or *ACX5* (Cruz Castillo et al., 2004; Afitlhile et al., 2005; Li et al., 2005; Pinfield-Wells et al., 2005; Schilmiller et al., 2007). JA is important for male reproductive function in Arabidopsis, with roles in production of viable pollen, filament extension, and correct timing of anther dehiscence (Sanders et al., 2000; Stintzi and Browse, 2000). Unlike many JA biosynthetic and signaling mutants, however, *cts* mutants are male fertile. It is probable that the low residual levels of JA in *cts* mutants are sufficient for fertility because McConn and Browse (1996) used a triple fatty acid desaturase mutant with a leaky *fad7* allele to demonstrate the existence of a threshold limit for the JA precursor, linolenic acid (18:3), and by extension, JA itself, for male fertility. Intriguingly, it has been reported that the double-mutant *ped1,ped3-1*, which lacks both *CTS* and *KAT2*, is sterile (Hayashi et al., 2002), perhaps because the compound effect of mutating both genes reduces JA levels below the fertility threshold. The apparently ubiquitous expression of *CTS* and the known role of JA in male fertility led us to examine in more detail the roles of this transporter during postgerminative growth to determine what impact loss of *CTS* function has on vegetative and reproductive processes.

Fertilization in flowering plants is a multistep process that requires delivery of pollen sperm cells to the ovules, which are located deep within the flower (Johnson and Preuss, 2002). Lipid metabolism and signaling potentially play important roles in several stages of fertilization, starting with production and release of viable pollen (Sanders et al., 2000; Stintzi and Browse, 2000) and synthesis of the protein- and lipid-rich pollen coat, which has a protective function and provides essential signals for binding, recognition, and hydration by the stigma (Zinkl et al., 1999). Deficiencies in pollen coat lipids lead to hydration defects and conditional male sterility in Arabidopsis *cer* mutants (Preuss et al., 1993; Fiebig et al., 2000) and pollen coat lipids have also been suggested to direct a water gradient, which is required for organization of cell polarity prior to germination (Wolters-Arts et al., 1998).

There are many parallels between pollen and seeds: both are dispersal units, which germinate to produce polarized, tip-growing structures, the pollen tube and the radicle, respectively. Both structures contain abundant presynthesized mRNAs, which are translated upon germination (Dure and Waters, 1965; Mascarenhas, 1993), although the biochemical mechanisms and regulation of pollen and seed germination appear to be different. In common with oilseeds, mature pollen of many species accumulates lipids (Baker and Baker, 1979). As in seeds, storage lipids take the form of TAGs, which are stored together with phospholipids and oleosins in oil bodies (Kuang and Musgrave, 1996; Kim et al., 2002). During pollen formation, oleosins and TAGs are synthesized in the abundant endoplasmic reticulum of tapetosomes and numerous oil droplets are produced in a manner apparently identical to that of seeds (Hsieh and Huang, 2005). Ultrastructural studies have reported the presence of numerous peroxisomes and lipid bodies in Arabidopsis pollen (Van Aelst et al., 1993). Synthesis of lipid bodies occurs shortly after pollen mitosis I and is restricted to the vegetative cell (Park and Twell, 2001). Microbodies, mitochondria, and lipid droplets are present in close spatial association in late pollen development and these organelles are also present in mature pollen, but they are more dispersed (Kuang and Musgrave, 1996). Similarly, pollen of the closely related species, oilseed rape (*Brassica napus*), has been shown to contain polymorphic microbodies, which were often in contact with lipid bodies, consistent with a function in lipid catabolism (Charzynska et al., 1989). In olive (*Olea europea*), oil bodies disappear following pollen germination (Rodriguez-Garcia et al., 2003) and, accordingly, a putative TAG lipase (*SUGAR-DEPENDENT1* [*SDP1*]-*like*), which is abundantly expressed in pollen and flowers, has recently been identified in Arabidopsis (Eastmond, 2006). By analogy with *SDP1*, this gene might encode a TAG lipase involved in the mobilization of pollen oil reserves.

Following germination, the pollen tube must penetrate the cell wall of the stigma and grow into the style and transmitting tract. Pollen tubes grow at extremely high rates in vivo and consequently have a very high demand for energy (Lord, 2000; Lord and Russell, 2002). Because pollen is often in excess, tubes must compete for access to ovules; thus, rapid growth is key to male reproductive success (Howden et al., 1998). The pollen tube is known to interact intimately with the nutrient-rich extracellular matrix of the stylar tract and adhesion factors implicated in pollen tube growth have been identified in lily (*Lilium longiflorum*) and tobacco (*Nicotiana tabacum*; Lord, 2000, 2003; Lord and Russell, 2002). Pollen tube growth and guidance are separable genetically (Johnson et al., 2004), but the identities of diffusible signals directing pollen tubes to the ovules and ultimately permitting entry to the micropyle have remained elusive until recently (Johnson and Preuss, 2002; Higashiyama et al., 2003; McCormick and Yang, 2005). However, recent studies

have implied that γ-aminobutyric acid (GABA) and NO are directional signals participating in pollen tube guidance in Arabidopsis (Palanivelu et al., 2003; Prado et al., 2004).

In this study, we have examined the postgerminative phenotype of *cts* mutants, with particular attention to fertility. We present data demonstrating that fertilization is compromised in *cts* and other β-oxidation mutants and that this defect is due to impaired pollen germination and reduced elongation of pollen tubes, rather than reduced JA or IAA biosynthesis. Although a role in processing of unknown signals cannot be ruled out, we propose that an important function of β-oxidation in fertilization is the provision of energy for pollen tube germination and growth via mobilization of TAG in both male and possibly also female tissues.

RESULTS

Postgerminative Phenotype of *cts* Mutants

Following mechanical rupture of the testa and seedling establishment in the presence of exogenous Suc, *cts* mutants can be transferred to soil and complete the life cycle (Russell et al., 2000; Footitt et al., 2002). However, mutation of *CTS* had subtle effects on vegetative growth: Rosette leaf number and area were reduced in *cts-1* mutants compared to the wild type, Landsberg *erecta* (L*er*), but these parameters were much less affected in *cts-2* mutants, which more closely resembled the wild type, Wassilewskija2 (Ws2; Supplemental Fig. S1). Cauline leaf number and area were also reduced in *cts-1* (Supplemental Fig. S1). Despite the reduction in leaf tissue in *cts-1*, there was no significant effect on photosynthesis in either of the mutant alleles (data not shown).

Although *cts* mutants were fertile, both *cts-1* and *cts-2* exhibited an altered reproductive phenotype. Time to bolting was not greatly affected, but both *cts-1* and *cts-2* alleles produced more flowers on the primary inflorescence than their respective wild types, L*er* and Ws2 (Fig. 1A). Floral development was examined in the *cts* mutants: Mutant flowers appeared morphologically normal, with the exception that extension of the filaments was transiently delayed. Observation of flowers at stage 13 revealed that the ratio of long stamen to pistil length of the *cts* mutants was approximately 90% of the wild-type ratio (Fig. 1, B and C). This ratio was restored in *cts-1* plants expressing the *CTS* open reading frame (ORF) under the control of the native *CTS* promoter (Fig. 1C). Filament extension in mutant flowers increased in subsequent developmental stages such that no difference between wild type and mutants was distinguishable. By floral stage 14, mutant anthers had extended above the stigma, permitting the deposition of pollen. Pollen of *cts* mutants was 100% viable, as judged by vital staining (data not shown).

A possible biochemical basis for delayed filament extension was investigated by application of hormones to *cts* flower buds. Whereas painting buds with JA did not affect filament extension (data not shown), this parameter was enhanced by spraying with the synthetic auxin analog, 1-naphthaleneacetic acid (NAA), such that treated mutants resembled the wild type (Fig. 1D). Exogenous IAA also increased extension of *cts* filaments, but a higher concentration was required (50 μM; data not shown), perhaps because IAA is less permeant and less stable in planta than NAA (Delbarre et al., 1996).

Fertilization Is Reduced in *cts* Mutants

Following pollination, there was an approximate 50% increase in silique production in plants of both mutant alleles in comparison to their respective wild types (Fig. 2A). Total silique dry weight was unchanged in mutants, but mutant siliques were shorter than wild type (Fig. 2, B and C). Shorter siliques were associated with reduced fertilization of ovules and also increased abortion of embryos in mutant alleles (Fig. 3, A–C). The percentage of fertilized ovules was reduced from 92% in L*er* to 76% in *cts-1* and from 90% in Ws2 to 57% in *cts-2*. Wild-type levels of fertilization were restored by genetic complementation (Fig. 3A). To test whether the fertility defects were specific to *cts* mutants or reflected a more general defect in β-oxidation, fertilization was assessed in the single *kat2-1* and the *lacs6-1*, *lacs7-1* double mutants, which are deficient in KAT, and two peroxisomal long-chain acyl-CoA synthetases, respectively (Germain et al., 2001; Fulda et al., 2004). The percentages of fertilized ovules and aborted seeds in *kat2-1* were similar to those in *cts* alleles, whereas the reduction in fertilization in *lacs6-1*,*lacs7-1* was intermediate between that of *cts* alleles and wild type (Fig. 3B).

To investigate whether reduced fertility was a consequence of the reduced jasmonate levels found in *cts* mutants (Theodoulou et al., 2005), JA and its precursors, α-linolenic acid and OPDA, were painted onto the apical buds of the primary inflorescence of *cts* mutants and wild types. Application of α-linolenic acid did not affect wild types, but proved to be toxic to *cts* plants, causing scorching of flower buds, which is suggestive of impaired fatty acid metabolism in mutant flowers. JA and OPDA induced stunting of the inflorescence and shortening of siliques of *cts* plants, with no recovery of fertility. Wild-type plants were unaffected and application of the wetting agent, Tween 20, had no effect in any genotype (data not shown). Similarly, we tested whether fertility could be restored by application of exogenous auxin, but found no effect on fertilization following spraying of flower buds with 10 μM NAA.

cts Pollen Performs Less Well Than Wild-Type Pollen

Reduced fertility could be due to defects in either sporophytic tissue or in gametophytic tissue. To test

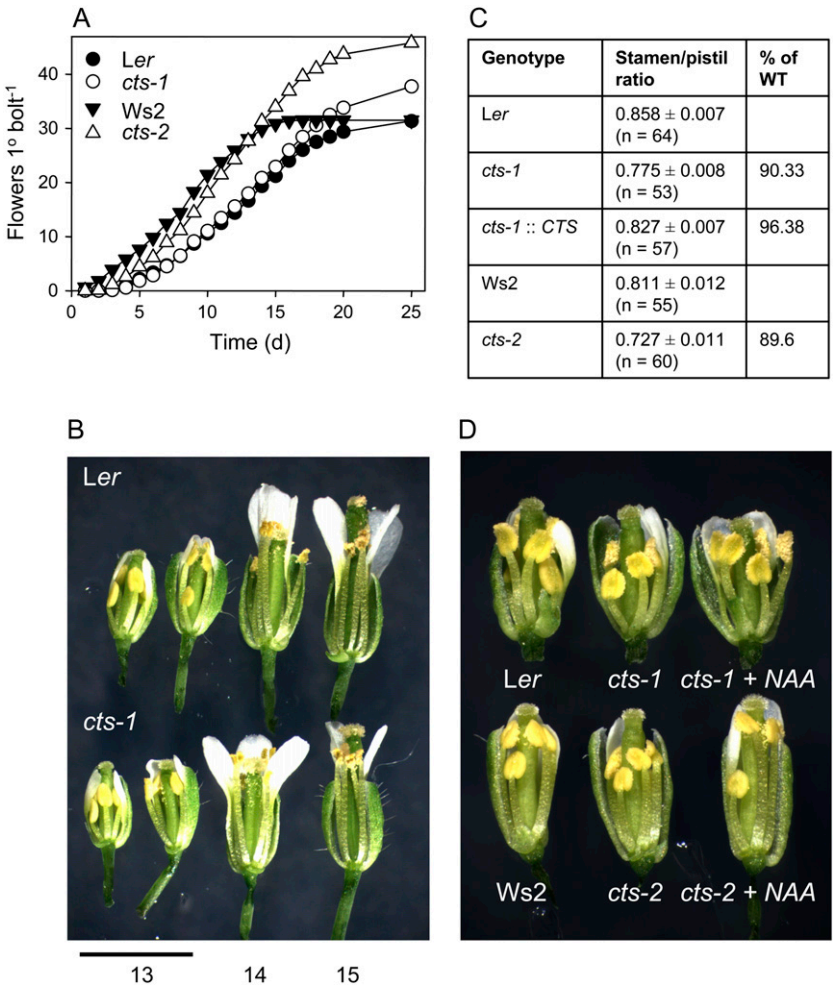

Figure 1. Floral phenotype of *cts* mutants. A, Cumulative flowering on the primary inflorescence (n = 23–32). B, Photograph of partially dissected flowers (stages 13–15) from primary inflorescences of Arabidopsis, accession L*er*, and the *cts-1* mutant. C, Ratio of long stamen/pistil lengths in different genotypes, including *cts-1* plants transformed with a CTS promoter-cDNA cassette (*cts-1*::*CTS*). Values are means ± SE. D, Photograph of partially dissected flowers (stage 13) of wild-type (L*er*, Ws2), *cts* mutants, and *cts* plants treated with 10 μM NAA.

for gametophytic effects of the *cts* mutation, plants heterozygous for *cts-1* or *cts-2* were allowed to self-fertilize and the genotypes of progeny were deduced by seed germination assays and allele-specific PCR. A significant deviation from the Mendelian ratio of 1:2:1 (wild type:+/*cts*:*cts*/*cts*) was observed (Table I), suggesting reduced transmission of mutant alleles (Howden et al., 1998). Therefore, the performance of gametes bearing the *cts* mutation was tested further in a series of reciprocal crosses between plants heterozygous for the *cts-1* or *cts-2* mutation and those homozygous for wild-type or mutant alleles. All crosses produced seeds that were subjected to germination assays and the genotype determined by PCR. The expected 1:1 segregation ratio of genotypes was observed in progeny of crosses between a heterozygous *cts*/+ female parent with either the wild type or with the homozygous mutant as the male parent (Table II). However, a highly significant deviation from this ratio was observed where heterozygous *cts*/+ pollen was used to pollinate wild-type or *cts*/*cts* pistils: approximately 5:1 for *cts-1*/+ pollen and 10:1 in the case of the *cts-2*/+ pollen ($P < 0.001$). Thus, transmission through the male, but not the female, gametes was affected (Table II). This result strongly suggests that, in a competitive situation, *cts-1* and *cts-2* pollen tubes are less able to target ovules than the respective wild types. The transmission efficiencies of *cts* alleles were also used to calculate the expected proportion of mutant progeny of selfed heterozygotes (Park et al., 1998), which agreed well with the observed values (Supplemental Table S1).

Pollen Tube Growth Is Impaired in *cts* Mutants

In *cts* mutants, unfertilized ovules appeared to predominate toward the base of the silique (Fig. 3C), suggesting a possible defect in pollen tube growth. Therefore, emasculated flowers of *cts-1* and L*er* were self- and cross-pollinated and pollen germination on the pistil allowed to proceed for 24 h. Subsequent aniline blue staining of callose in the pollen and pollen tube cell wall indicated that fewer *cts-1* pollen tubes reached the base of *cts-1* pistils when compared to L*er* pollen inoculated onto both mutant and wild-type pistils. Furthermore, a higher proportion of *cts-1* pollen tubes appeared to target ovules nearer to the stigmatal end of *cts-1* pistils. In contrast, pollen tube growth

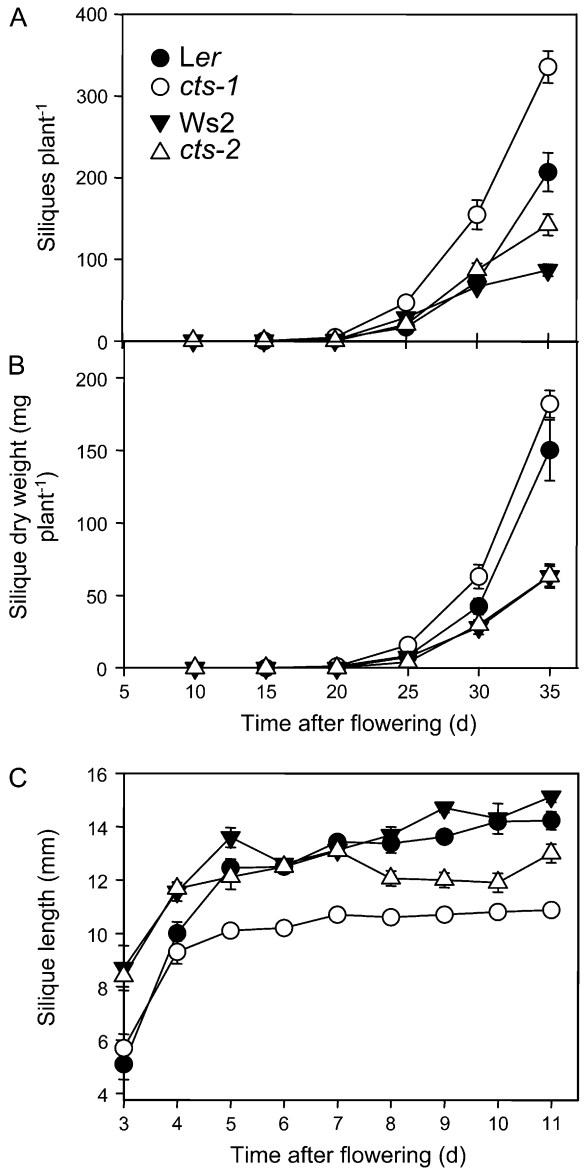

Figure 2. Silique production in *cts* mutants and respective wild types. A, Total silique production. B, Total silique dry weight. C, Silique length. Black circles, L*er*; white circles, *cts-1*. Black triangles, Ws2; white triangles, *cts-2*. Values are means ± SE (n = 10–17).

appeared normal when *cts-1* pollen was inoculated onto wild-type pistils (Fig. 4A), indicating that the presence of CTS in either male or female tissues was sufficient for wild-type pollen tube growth. Reduced pollen tube growth was also observed in selfed *cts-2* compared to crosses in which one parent was wild type, Ws2 (data not shown). Pistils were of similar length in both wild types and mutants (Fig. 4B).

Peroxisomes Are Abundant in Many Floral Tissues

Because aniline blue staining indicated a role for CTS in both male and female tissues, we investigated the abundance of peroxisomes—the organelles that house the β-oxidation pathway—in different floral tissues. Peroxisomes were visualized by confocal laser-scanning microscopy in reproductive tissues from Arabidopsis plants expressing a peroxisomal-targeted GFP reporter (Cutler et al., 2000). Peroxisomes were abundant and readily detected in petal, sepal, pistil, and anther (Fig. 5, A and B; data not shown). However, expression of the GFP reporter was not detected in in vitro-germinated pollen (Fig. 5C), developing pollen grains within the anther locules (Fig. 5B), or pollen on the stigmatal surface (data not shown). Peroxisomes were abundant within the wall of the gynoecium (Fig. 5D) and within the transmitting tract tissue (Fig. 5, D and E). Peroxisomes were also present within the funiculus and fertilized ovules (Fig. 5F).

GABA Metabolism in Flowers of *cts* and *kat2-1* Mutants

Because GABA is believed to be required for correct pollen tube growth and targeting (Palanivelu et al., 2003), we tested the hypothesis that CTS and, by extension, β-oxidation, might play a role in GABA catabolism, thereby assisting the generation of a GABA gradient in reproductive tissue, which permits optimal fertility. GABA and GABA shunt metabolites (Fig. 6A) were measured in flowers of both L*er* and Ws wild types and in flowers of *cts* and *kat2-1* mutants. Ws2 flowers contained significantly less GABA than those harvested from L*er* plants (Fig. 6B). GABA content was significantly reduced in both *cts-1* and *cts-2*, but not *kat2-1*, relative to wild type. Of the catabolites, γ-hydroxybutyrate (GHB) content appeared to be elevated in *kat2-1*, but levels were not significantly different in any of the genotypes tested. Succinate content was reduced in flowers of both *cts-1* and *kat2-1*, but not in *cts-2*. Succinic semialdehyde, which is highly reactive, could not be detected, in agreement with a previous report (Palanivelu et al., 2003).

A defect in the first step of GABA catabolism in the *pop2-1* mutant leads to GABA hypersensitivity of pollen tube growth (Palanivelu et al., 2003; Fig. 6A). The GABA sensitivity of in vitro-grown *cts* and *kat2-1* pollen tubes was therefore tested over a range of GABA concentrations from 0 to 1 mM, as described in Palanivelu et al. (2003), but was found to be highly variable (data not shown).

In Vitro Pollen Germination and Tube Growth Are Impaired in β-Oxidation Mutants in the Absence of Suc

To investigate further the defect in the performance of *cts* mutant pollen, we measured pollen germination and tube growth in vitro. Pollen was germinated in isoosmotic medium containing 18% polyethylene glycol (PEG) or 16% PEG plus 2% Suc. The percentage of wild-type pollen germination varied between experiments, as has been reported previously for Arabidopsis (Johnson-Brousseau and McCormick, 2004); however, when pollen was germinated in the absence of an

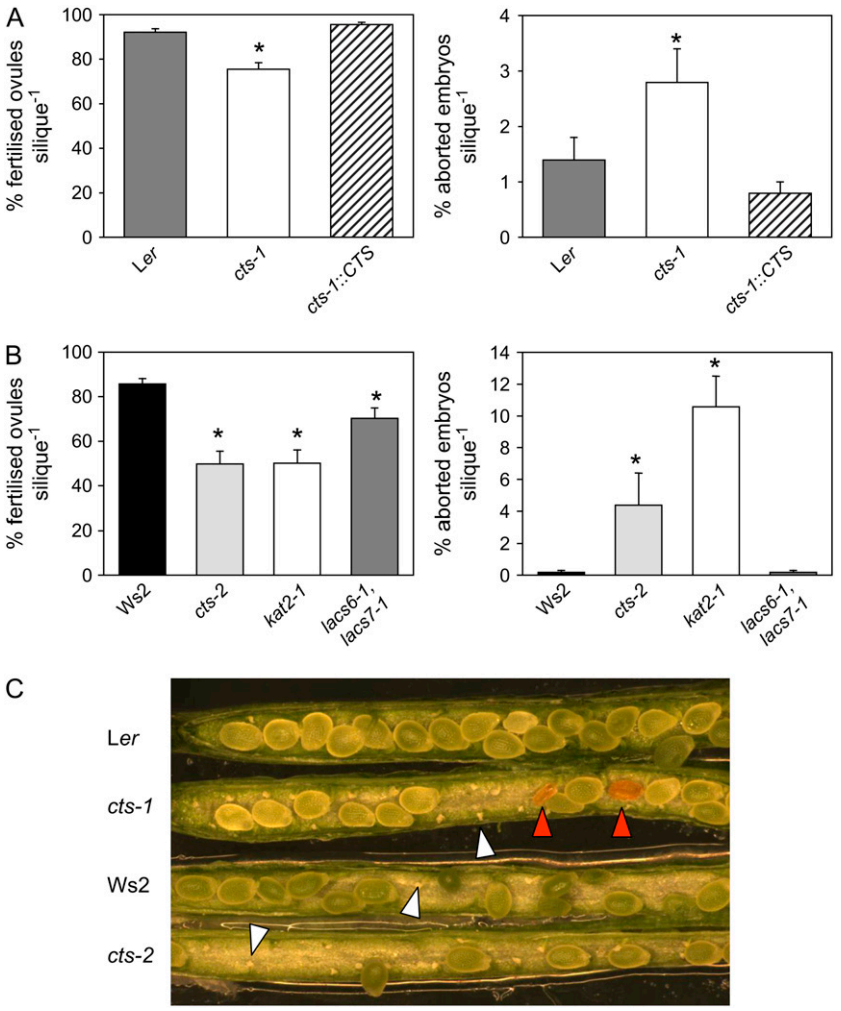

Figure 3. Fertilization in *cts* mutants. A, Percentage fertilized ovules and aborted embryos in siliques of L*er*, *cts-1*, and *cts-1* plants transformed with a CTS promoter-cDNA cassette (*cts-1*::*CTS*). Siliques were harvested 10 DAF. Values are means ± SE (n = 10–15 siliques). B, Percentage fertilized ovules and aborted embryos in siliques of wild type, Ws2, and β-oxidation mutant alleles: *cts-2*, *kat2-1*, *lacs6-1*,*lacs7-1*. The Ws4 wild-type behaved identically to Ws2 (data not shown). Values are means ± SE (n = 11–18 siliques). Asterisks in A and B denote significant differences to wild type at 5%, as determined following ANOVA, using the LSDs on the transformed data. C, Dissected siliques (harvested 10 DAF) showing unfertilized ovules (white arrowheads) and aborted embryos (red arrowheads) in *cts* mutants.

exogenous carbon source, we consistently observed a reduction in pollen germination (of between 40% and 65%) in *cts* and *kat2-1* mutants (Fig. 7A). When Suc was included in the germination medium, the percentage germination increased in all genotypes and there was no significant difference between mutants and their respective wild types, with the exception of *kat2-1*. Similarly, we also investigated pollen tube growth in vitro in the presence and absence of a carbon source. L*er* pollen tubes were of similar length in both media, but Ws2 pollen tubes tended to grow longer in the presence of Suc (Fig. 7B). In Suc medium, pollen tubes of the β-oxidation mutants, *cts-1*, *cts*-2, and *kat2-1*, were of similar length to those of the respective wild types. However, mutants produced significantly shorter pollen tubes in the absence of a carbon source. *cts-1* plants complemented with the *CTS* genomic clone produced pollen tubes of wild-type length, confirming that loss of *CTS* function is responsible for the defect in pollen tube growth. Inclusion of auxin in the medium did not promote germination or tube growth of either mutant or wild-type pollen (data not shown).

Table I. *Genetic transmission of cts alleles*

Observed frequency of different genotypes in self-progeny of +/*cts* heterozygotes is shown.

Self-Progeny of +/*cts*		
+/+	+/*cts-1*	*cts-1*/*cts-1*
123	67	21
+/+	+/*cts-2*	*cts-2*/*cts-2*
95	106	17

DISCUSSION

cts Mutants Have a Subtle Vegetative and Floral Phenotype

Once *cts* plants had developed photosynthetic competence, the lack of *CTS* had only a minor effect on vegetative growth, with reduced leaf number and area in *cts-1*, but not *cts-2* (Supplemental Fig. S1). This is in

Table II. *Offspring ratios and transmission efficiencies determined from reciprocal crosses of plants carrying cts-1, cts-2, and CTS alleles*

P, Probability of obtaining the observed variation from an expected 1:1 ratio by chance. *P* was calculated using GenStat (χ^2 test). Transmission efficiency (TE) represents the fraction of mutant gametes that successfully transmit the mutation (Howden et al., 1998) and is defined as TE (%) = (no. mutants)/(no. wild-type plants) × 100.

♀ Parent	♂ Parent	No. Offspring			Probability	TE
		+/+	*cts-1*/+	*cts-1*/*cts-1*		
+/+	*cts-1*/+	97	21		$P < 0.001$	21.6%
cts-1/+	+/+	49	46		$P > 0.05$	93.9%
cts-1/+	*cts-1*/*cts-1*		118	140	$P > 0.05$	
cts-1/*cts-1*	*cts-1*/+		159	31	$P < 0.001$	
		+/+	*cts-2*/+	*cts-2*/*cts-2*		
+/+	*cts-2*/+	107	11		$P < 0.001$	10.3%
cts-2/+	+/+	60	68		$P > 0.05$	111.3%
cts-2/+	*cts-2*/*cts-2*		72	56	$P > 0.05$	
cts-2/*cts-2*	*cts-2*/+		151	15	$P < 0.001$	

agreement with the phenotype reported for the *pxa1* allele (Zolman et al., 2001) and the assertion that β-oxidation of fatty acids does not play a prominent role in vegetative growth of the unstressed plant, although it is likely to serve a housekeeping function in membrane lipid turnover (Graham and Eastmond, 2002). The loss of *CTS* function was more pronounced during reproductive development: Both *cts* mutants produced more flowers and also more siliques than wild types. This may represent a compensation mechanism, whereby reduced seed set in individual siliques is offset by increased total silique production. Of the known β-oxidation mutants, only *abnormal inflorescence meristem 1* (*aim1*), which is deficient in a MFP, has a reported floral/fertility phenotype (Richmond and Bleecker, 1999). However, compared to *aim1* mutants, which exhibit severe defects in floral development and are practically sterile, the *cts* mutant has a much more subtle reproductive phenotype. Unlike *aim1* (Richmond and Bleecker, 1999), floral morphology was normal in *cts* mutants, although they exhibited delayed filament extension (Fig. 1B), a phenotype that is shared by plants lacking functional allene oxide synthase and COI1, which are required for JA biosynthesis and perception, respectively (Feys et al., 1994; Park et al., 2002; von Malek et al., 2002). However, application of JA did not rescue the anther extension phenotype of *cts* flowers (data not shown). Delayed filament extension has also been reported for mutants in which auxin transport or signaling is perturbed; for example, the filaments of the auxin response transcription factor mutants *arf6-2* and *arf8-3* were shorter than those of wild-type stage 12 flowers, although they elongated further as the flowers matured, as was the case for *cts* alleles (Nagpal et al., 2005). Accordingly, application of 10 μM NAA to *cts* flower buds resulted in wild-type filament extension (Fig. 1D). This result is consistent with a role for CTS in the conversion of IBA to IAA, although it is possible that exogenously applied auxin bypasses a requirement for CTS unrelated to auxin metabolism.

Fertilization Is Impaired in β-Oxidation Mutants

A noticeable feature of the *cts* mutants was the production of shorter siliques (Fig. 2), a phenotype that is often indicative of reduced fertilization, as, for example, in the JA biosynthetic mutant, *opr3* (Stintzi and Browse, 2000), and mutants lacking the VANGUARD pectin methylesterase, which is required for pollen tube growth (Jiang et al., 2005). Dissection of siliques revealed that fertilization was reduced in both *cts-1* and *cts-2*, with a more marked effect in the latter allele (Fig. 3, A and B). Fertilization was also compromised in *kat2-1* and, to a lesser extent, in the *lacs6-1*,*lacs7-1* double mutant (Fig. 3B), implying a general role for β-oxidation in fertilization. Although *cts-1* was complemented by a *CTS* promoter-ORF construct (Fig. 3A), *cts* mutants were not rescued by exogenous application of JA or its precursors, 18:3 and OPDA, demonstrating that the fertilization phenotype does not result from JA deficiency. Application of auxin also failed to rescue the *cts* fertilization defect, which is perhaps not surprising, given that Arabidopsis flowers contain abundant IAA and IAA conjugates (Aloni et al., 2006) and that conversion from IBA may only account for a relatively small proportion of the free IAA pool (Bartel et al., 2001). The fertility phenotype of *lacs6-1*, *lacs7-1* was intermediate between that of *cts* mutants and *kat2-1*, suggesting that full fertility requires β-oxidation of a substrate that is handled by additional or alternative acyl-activating enzymes.

Pollen Tube Germination and Growth Are Impaired in *cts* Mutants

Whereas *CTS* is expressed ubiquitously, it should be noted that transcripts are not expressed preferentially in either male or female gametophytic tissues or in specific sporophytic tissues (Becker et al., 2003; Honys and Twell, 2003, 2004; Wellmer et al., 2004; Yu et al., 2005). Thus, CTS could play a role in either or both sporophytic or gametophytic tissues. Genetic analysis

was undertaken to determine whether the fertility phenotype of *cts* mutants arises from a gametophytic defect. The genotypes of progeny obtained from selfed heterozygotes deviated from the Mendelian 1:2:1 ratio, the low frequency of *cts* homozygotes observed among +/*cts* self-progeny indicating strongly reduced transmission of *cts* (Howden et al., 1998). In reciprocal cross experiments, only 21.6% of pollen carrying *cts-1* successfully transmitted the mutation and this effect was more severe in *cts-2* (transmission efficiency 10.3%) in agreement with the observation that *cts-2* plants exhibited greater reduction in fertilization than *cts-1* (Fig. 3). However, when the pistil was heterozygous for *cts*, mutant pollen tubes did not discriminate between wild-type and mutant eggs and female transmission efficiency of *cts* alleles was close to 100% (Table I), indicating that expression of *CTS* in the female gametophyte does not contribute to fertilization.

In accordance with a role for *CTS* in pollen, aniline blue staining revealed that in vivo pollen tube growth was impaired when *cts-1* and *cts-2* mutants were selfed (Fig. 4A). However, this defect was not observed when only one parent bore the *cts* mutation, suggesting that *CTS* can support pollen tube growth when expressed in either the pollen or the female tissue alone. Taken together with the genetic analysis, this not only suggests that expression of *CTS* in pollen is important for pollen tube growth, but also indicates that *CTS* has a function in female sporophytic tissue that can contribute to this process. The presence of peroxisomes within the transmitting tract is consistent with a role for CTS in this tissue (Fig. 5E). Abundant peroxisomes were also visualized in petals, sepals, funiculus, and ovules, but were not observed in pollen of *35S::GFP-MFP2* plants (Fig. 5). This reflects the properties of the *35S* promoter, which is known not to be active in Arabidopsis pollen (Wilkinson et al., 1997). It should be noted, however, that pollen does indeed contain abundant peroxisomes, as revealed by studies using the pollen-specific *LAT52* promoter to drive expression of a peroxisomally targeted enhanced cyan fluorescent protein in lily (Prado et al., 2004) and the *ACX1* promoter to drive expression of a peroxisomal enhanced yellow fluorescent protein in Arabidopsis (Schilmiller et al., 2007).

Given the numerous functions of β-oxidation in plants (Baker et al., 2006), *CTS* could play more than one role in fertilization, including synthesis of JA, synthesis of IAA, provision of energy and carbon skeletons via lipid catabolism, or processing of a signal that affects pollen tube growth and guidance. Therefore, the biochemical basis for impaired pollen germination and tube growth was investigated using pollen germinated in vitro. Neither pollen germination nor tube growth was promoted by auxin (data not shown), which is in agreement with the inability of auxin to restore fertilization in *cts* mutants. Pollen germination in the absence of exogenous Suc was impaired in *cts* and other β-oxidation mutants, although this was a moderate effect compared to the severe seed germination phenotype of *cts* alleles (Russell et al., 2000; Footitt et al., 2006). In the absence of an exogenous carbon source, pollen tubes of *cts* and other β-oxidation mutants were shorter than their respective wild types, but this difference was not apparent when Suc was included in the medium (Fig. 7). These findings are consistent with the suggestion that catabolism of stored lipids during pollen maturation could provide sugars to support pollen germination and tube growth.

Pollen tubes grow at a higher rate in vivo than in vitro (Johnson and Preuss, 2002), suggesting that nutrients and/or signals from the stylar tissue contribute to growth through the transmitting tissue (Lord, 2000; Lord and Russell, 2002). Pollen represents a symplastically isolated sink, which must take up sugars from the apoplast, and pollen grains and tubes consequently express several plasma membrane-bound transporters, which mediate uptake of sugars and other nutrients at different stages of growth and development (Stadler et al., 1999; Truernit et al., 1999; Schneidereit et al., 2003, 2005; Scholz-Starke et al.,

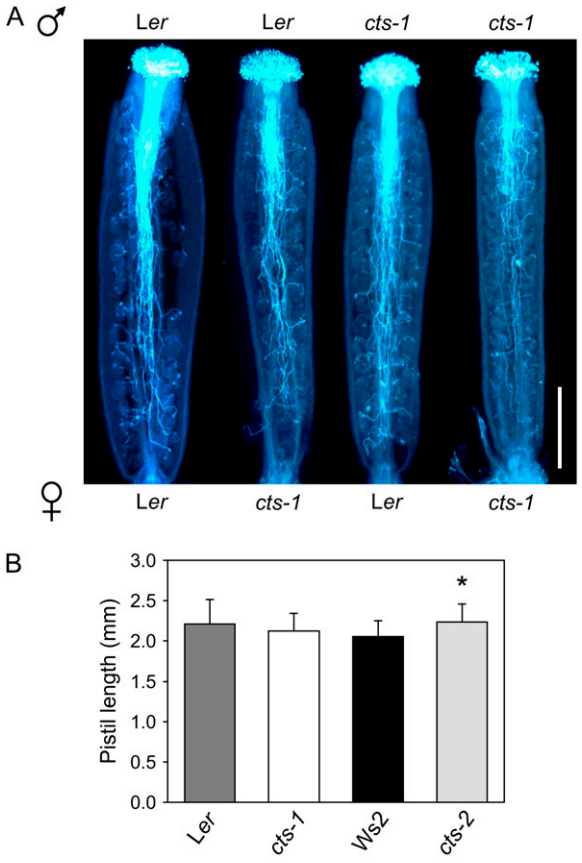

Figure 4. Pollen tube growth in *cts* mutants and respective wild types. A, Pollen tube growth in mutant and wild-type pistils was determined in self- and cross-pollinated pistils. Pistils were removed 24 h after pollination and stained with aniline blue. Bar = 500 μm. B, Pistil lengths of L*er*, *cts-1*, Ws2, and *cts-2* flowers (stage 14). Values are means ± SE (n = 22–28). Asterisk denotes that *cts-2* is significantly different than Ws2 at 2%, as determined by ANOVA.

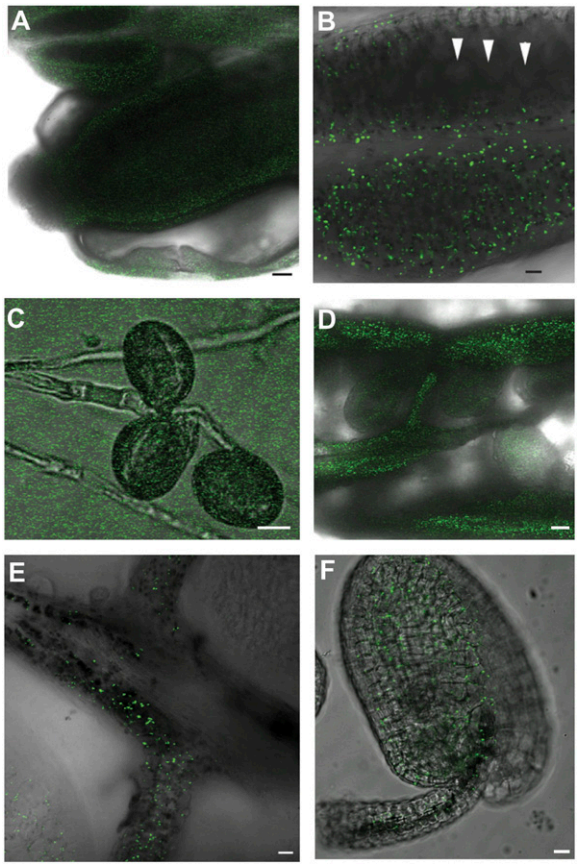

Figure 5. Peroxisomes in reproductive tissues of Arabidopsis. Peroxisomes were visualized by confocal laser-scanning microscopy of plants expressing peroxisomally targeted enhanced GFP. A, Stage 12 flower showing GFP expression in petal, stamens (anther and filament), and pistil. Scale bar = 50 μm. B, Close-up of anther from stage 12 flower showing lack of expression in developing pollen grains (arrowheads) within the anther. Scale bar = 20 μm. C, Pollen germinated 4 h in vitro in Suc-containing medium, showing lack of expression. Scale bar = 10 μm. D, Dissected silique from stage 16 showing peroxisomes in the gynoecium wall and transmitting tissue. Scale bar = 50 μm. E, Higher magnification image showing peroxisomes within the transmitting tissue and funiculus. Scale bar = 10 μm. F, Peroxisomes in the fertilized ovule and funiculus from a stage 16 flower. Scale bar = 10 μm.

2003; Bock et al., 2006). Interestingly, *hap3*, an Arabidopsis mutant with short pollen tubes, has been attributed to a T-DNA insertion in the *SUC1* gene, which encodes a Suc transporter expressed specifically in male tissues (Stadler et al., 1999; Johnson et al., 2004). This implies that the female sporophytic tissue plays a role in supporting pollen tube growth energetically, as suggested by aniline blue staining (Fig. 4A). β-Oxidation of fatty acids could serve to provide Suc via the glyoxylate cycle and gluconeogenesis, as is the case in germinating oilseeds, or, alternatively, could provide acetyl equivalents for respiration.

There is some disagreement in the literature as to whether the glyoxylate cycle operates in growing pollen tubes, dependent on the species examined (Zhang et al., 1994; Mellema et al., 2002). However, examination of microarray data indicates that transcripts encoding the glyoxylate cycle enzymes isocitrate lyase and malate synthase are virtually absent from pollen and indeed also from other floral tissues in Arabidopsis (Zimmermann et al., 2004). Reporter fusions and reverse transcription-PCR studies also demonstrated lack of expression of malate synthase in floral tissue, whereas KAT (*PED1/KAT2*) is highly expressed (Charlton et al., 2005). In contrast, peroxisomal citrate synthase (*CSY2* and *CSY3*) transcripts are expressed throughout the flower (Zimmermann et al., 2004), suggesting that lipids can be used as a source of carbon for respiration, as has been proposed for germinating sunflower (*Helianthus annuus*) seeds (Reymond et al., 1992). In this scenario, acetyl-CoA produced by β-oxidation is converted to citrate, which is exported from the peroxisome and participates in the citric acid cycle (Pracharoenwattana et al., 2005). Thus, products of fatty acid catabolism can pass from the peroxisome to the mitochondrion independently of the glyoxylate cycle, as is the case in Arabidopsis mutants, which lack isocitrate lyase (Eastmond et al., 2000).

An energetic role for CTS (and by extension β-oxidation) in female tissues is perhaps less obvious than in symplastically isolated pollen because it might be expected that sugars would be available from photosynthetic tissues of the flower. However, the ability of wild-type (but not *cts*) flowers to metabolize exogenously applied fatty acids suggests that maternal tissues are competent in lipid catabolism, and transcriptome data also support the assertion that flowers can potentially respire lipids. Moreover, the fact that the presence of CTS in either the male or the female tissues is sufficient for full fertility suggests that this transporter fulfills the same biochemical function in both pollen and the female sporophyte. It may be, however, that CTS plays an as-yet unknown role required for efficient pollen tube growth, which is unrelated to lipid catabolism.

β-Oxidation Is Not Essential for Correct Pollen Tube Guidance

Although the data presented in this manuscript support an energetic role for CTS in fertility, we did not rule out the possibility that β-oxidation might be involved in the production or removal of pollen tube guidance cues. Signals guiding the pollen tube to the female gametophyte are as yet relatively poorly characterized (Higashiyama et al., 2003), but it has been shown recently that a GABA transaminase encoded by *POP2* is required for correct pollen tube growth and targeting (Palanivelu et al., 2003). Because the GABA catabolite GHB, a short-chain hydroxy fatty acid, is a substrate for mitochondrial β-oxidation in mammals (Draye and Vamecq, 1987), we tested the hypothesis that *CTS*, and by extension, peroxisomal β-oxidation, might play a role in GABA catabolism in flowers.

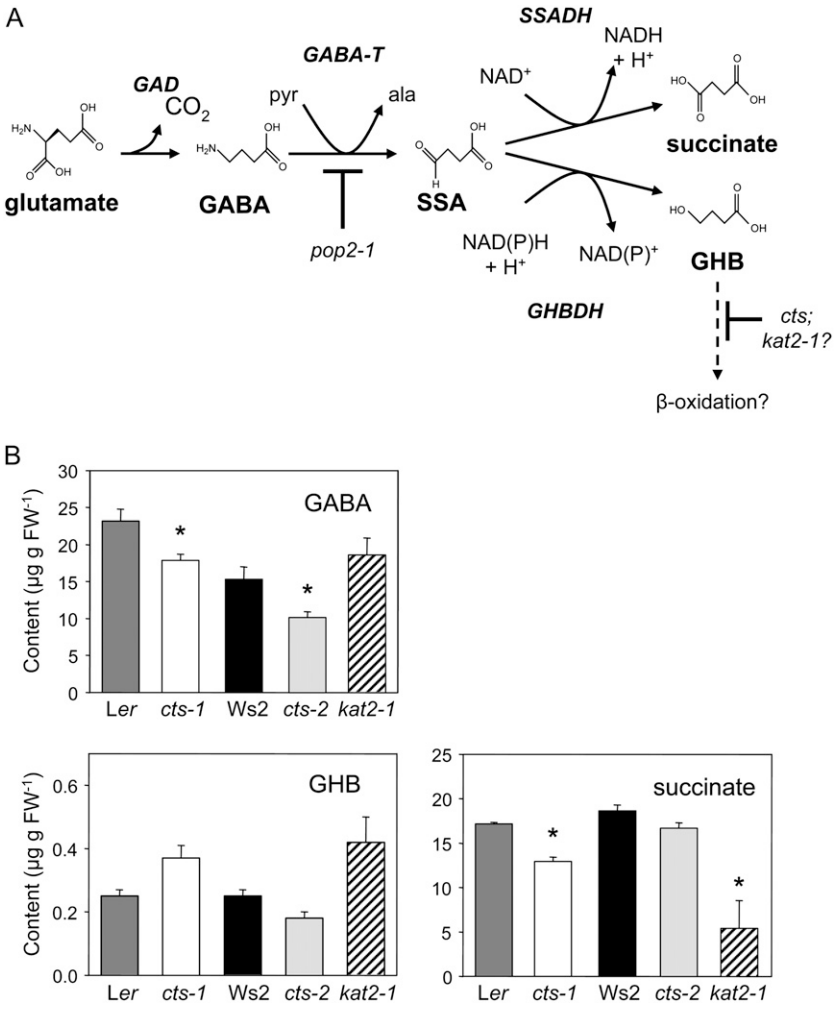

Figure 6. GABA and GABA shunt metabolite content in flowers. A, Proposed scheme for GABA metabolism in Arabidopsis flowers. GABA is synthesized in the cytosol from Glu by Glu decarboxylase (GAD). The first step in GABA catabolism is catalyzed by mitochondrial GABA transaminase (GABA-T). In Arabidopsis flowers, this enzyme is encoded by *POP2* and utilizes pyruvate (pyr) to yield Ala (ala) and succinic semialdehyde (SSA). Mutation of *POP2* results in elevated GABA. In mitochondria, SSA undergoes oxidation to succinate, catalyzed by succinic semialdehyde dehydrogenase (SSADH). Alternatively, under hypoxia, SSA is reduced via the cytosolic enzyme, γ-hydroxybutyrate dehydrogenase (GHBDH; also known as SSA reductase), to yield GHB. It is possible that GHB, a short-chain hydroxy fatty acid, is metabolized further by β-oxidation, by analogy with mammalian systems (the dotted line indicates that this step is hypothetical). β-Oxidation of GHB is potentially inhibited in *cts* and *kat2-1* mutants. B, Content of GABA, GHB, and succinate in Arabidopsis flowers. Values are means ± SE ($n = 4$). A *t* test was performed comparing every mutant and its respective wild type (L*er* or Ws2). Significant values ($P < 0.05$; critical *t* value 2.571) are indicated with an asterisk. Significant differences in GABA content were also found between the two Arabidopsis wild types.

In the *pop2* mutant, which lacks the first step of GABA catabolism, floral GABA levels are elevated approximately 100-fold (Palanivelu et al., 2003). Blocking metabolism of GHB by β-oxidation might therefore be predicted to increase GHB and possibly also GABA levels, thus perturbing the gradient, which is optimal for pollen tube growth and targeting. However, GHB levels did not show statistically significant differences in either *cts* or *kat2-1* flowers relative to wild type (Fig. 6B). Although GABA levels were decreased in flowers of *cts-1* and *cts-2*, the content of *kat2-1* flowers was unchanged. This suggests that, although there may be a minor contribution of *CTS* to GABA metabolism, any alteration in GABA content is unlikely to account for the reduced fertility that we observed in *cts* and *kat2-1* mutants. Moreover, unlike *pop2*, which is affected in both pollen tube growth and guidance, the effect of *cts* mutant alleles appears to be specific to pollen tube growth because we failed to detect any defective guidance in aniline blue-staining experiments (Fig. 4). The fact that a reduction, rather than an increase, in GABA was observed may reflect the complex posttranslational regulation of the pathway (Bouché and Fromm, 2004). Interestingly, succinate (the product of succinic semialdehyde dehydrogenase) was decreased in *cts-1* and *kat2-1* flowers. Succinate is produced by both the peroxisomal glyoxylate cycle and the mitochondrial citric acid cycle, but because the former pathway does not appear to operate in flowers (see above), reduced succinate in flowers of β-oxidation mutants most likely reflects the reduced flux of citrate from the peroxisome to the citric acid cycle.

CONCLUSION

The CTS ATP-binding cassette transporter plays a key role in regulating import of substrates into the peroxisome for β-oxidation. Although *CTS* was originally identified as a gene important for germination and seedling establishment, we show here that it is also required for full fertility in Arabidopsis. We have shown that *CTS* is required for efficient germination of pollen and that a defect in pollen tube growth is associated with reduced fertility of *cts* mutants, with *CTS* function in both male and female tissues contributing to pollen tube growth in vivo. We have tested three potential biochemical functions of CTS, synthesis

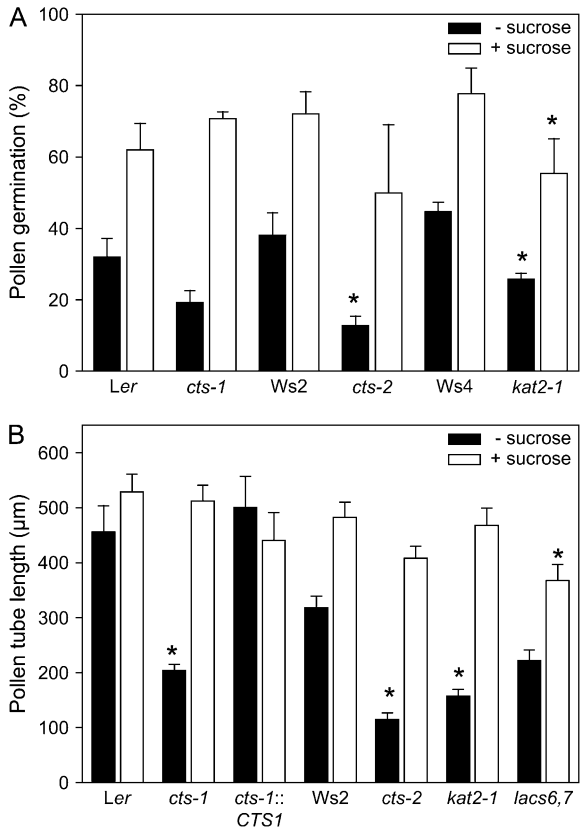

Figure 7. Pollen germination and tube growth in vitro. A, Pollen from stage 14 flowers was germinated in vitro in the presence or absence of a carbon source (2% Suc). Values are means ± SE of three independent slides (approximately 800 grains/slide). B, Pollen tube growth in different genotypes: L*er* (wild type), *cts-1*, and *cts-1* transformed with bacterial artificial chromosome clone 159N1 (*cts-1*::*CTS*); Ws2 (wild type), *cts-2*, *kat2-1*, and *lacs6-1*,*lacs7-1*. Pollen was germinated in vitro in the presence or absence of a carbon source (2% Suc) and pollen tube lengths of germinated pollen determined after 18 h. Values are means ± SE ($n = 50$). Asterisks denote significant differences from wild type at 5%, as determined following ANOVA, using the LSDs on the transformed data for A. Data presented in A and B are representative of several independent experiments.

of JA and IAA and catabolism of GABA, but did not find evidence that any of these functions underpin the fertilization phenotype of *cts* mutants. Although we cannot rule out a role for CTS (and β-oxidation) in processing an as-yet unidentified signaling molecule required for efficient fertilization, our data are consistent with the hypothesis that CTS contributes to fertilization via the provision of energy and carbon skeletons for the actively growing pollen tube.

MATERIALS AND METHODS

Plant Material

Isolation of *cts-1* and *cts-2* has been described previously (Footitt et al., 2002). Seeds of *kat2-1* and *lacs6-1*,*lacs7-1* were the kind gifts of Professor Steve Smith (University of Western Australia) and Dr. Martin Fulda (University of Göttingen), respectively.

Growth of Arabidopsis

Afterripened Arabidopsis (*Arabidopsis thaliana*) seeds of the mutants, *cts-1*, *cts-2*, and their respective wild types (L*er* and Ws2), were germinated as described in Footitt et al. (2006). After 7 d, germinated seedlings were transplanted to soil and plants grown to maturity in controlled environment rooms (16-h light at 23°C and 70% relative humidity/8-h dark at 18°C and 80% relative humidity). During the light phase, the incident photosynthetically active radiation was 150 to 175 μmol m^{-2} s^{-1} at the soil level. The position of plant trays was rotated to minimize light effects.

Floral and Fertilization Phenotypes

Cumulative flowering on the primary inflorescence was measured daily from the onset of flowering. Each day, flowering buds were marked by applying acrylic paint to the pedicel. Flowering was defined as first appearance of petals from within the enclosing sepals (stage 13; Smyth et al., 1990). At floral stage 13, sepals and petals were removed to expose the anthers and pistil. The ratio of long stamen/pistil length was recorded for each mutant and wild type. Anthers were also removed from flowers at stage 13 and pollen viability determined using Alexander's stain (Alexander, 1969). The incidence of fertilization and abortion of ovules in siliques derived from the primary inflorescence was determined at 5, 10, and 15 d after flowering (DAF). Unfertilized ovules appear white, whereas aborted ovules are shrunken and dark in color. Silique size was measured at increasing DAF.

The ability of JA (2.0 mM; Sigma) and its precursors OPDA (3.4 mM; Larodan AB), and α-linolenic acid (3.3 mM; Nu-Chek Prep) to rescue floral and fertilization phenotypes was tested by painting floral buds on the primary inflorescence daily. Solutions (including controls) contained 0.01% (w/v) Tween 20 as a wetting agent. Similarly, auxins (NAA and IAA) were applied to flower buds by spraying with a 10 μM aqueous solution.

Complementation of *cts-1*

A plant transformation vector, pG0229-T, was generated by transferring the terminator region of pUC18-spGFP6 (M. Suter-Grotemeyer and D. Rentsch, unpublished data) to the vector pGreenII0229 (Hellens et al., 2000). First, annealed oligonucleotides (5′-CTAGAGGATCCGCATG-3′ and 5′-CGGATCCT-3′) were ligated into the *Xba*I/*Sph*I sites of pUC18-spGFP6 to introduce a *Bam*HI site. The spacer-GFP terminator cassette of pUC18-spGFP6 was excised with *Kpn*I (made blunt with T4 DNA polymerase [New England Biolabs]) and *Sma*I and ligated into the *Not*I (blunt-ended)/*Sma*I sites of pGreenII0229. The spacer-GFP cassette was excised with *Bam*HI and the plasmid religated to yield pG0229-T.

A *CTS* promoter-ORF cassette was prepared in several stages. A promoter fragment corresponding to 2,638 to 1,445 bp upstream of the ATG was amplified with primers CTS ProFW2 (5′-GAGTACTTGGAAGAAGGCGG-TGA-3′) and CTS ProRV9 (5′-ATTGTACACCGCATGATTGAAGCACA-3′) and ligated into a blunted *Apa*I site of pBluescriptII SK− (Stratagene) to generate the plasmid, pSKPro-5′. A promoter fragment corresponding to 1,507 bp upstream and 38 bp downstream of the ATG was amplified with primers CTS ProFW4 (5′-GGAGTGATGTAATATGTACTTATCAGA-3′) and CTS ProRV (5′-CC**G**CG**G**CCCCGCTCAGTTAACTGCAATAG-3′; bold type indicates silent nucleotide changes to introduce the *Sac*II site) and ligated into the *Sma*I site of pBluescriptII SK−, to yield pSKPro-3′. The ORF of *CTS* was then amplified in two parts using cloned cDNA as template. The 5′ fragment amplified by primer CTS5FW (5′-GG**C**CG**C**GGTCTTGTAGCGTCAAGAC-GGA-3′; bold type indicates silent nucleotide changes to introduce the *Sac*II site) and CTS5RV (5′-GCCTTTGAATTAGTAGCAGATTCC-3′) and cloned in pCR-Blunt II Topo (Invitrogen) to yield pCRBlunt ORF5′, and the 3′ fragment amplified by primer CTS3FW2 (5′-GATCGGCAAAATGATGCGATGGT-3′) and CTS3RVwStop (5′-**CCCGGG**TCACTCTGTTGTCTGTTCGATCGA-3′; bold type indicates the introduced *Sma*I site) was restricted with *Pst*I and ligated in the *Pst*I/*Eco*RV sites of pBluescriptII SK−, to generate pSK ORF3′. The promoter and the 5′ portion of the *CTS* ORF were assembled in a three-way ligation between pSK Pro-5′ restricted with *Bbs*I/*Pst*I, pSK Pro-3′ restricted with *Bbs*I/*Sac*II, and pCRBlunt ORF5′ restricted with *Sac*II/*Pst*I, to give pSK ProORF5′. The 3′ portion of the CTS ORF was excised from pSK ORF3′ with *Pst*I/*Sma*I and cloned in the corresponding sites of pSK ProORF5′ to yield pSK ProORF. Finally, the promoter-ORF cassette was excised with *Kpn*I/*Sma*I and ligated into the corresponding sites of pG0229-T. The construct pG0229-T/*CTS* prom-ORF was introduced into *cts-1* plants by

Agrobacterium-mediated transformation. Seeds of transformed plants were sown in soil and after the appearance of the first two true leaves sprayed repeatedly with a 150-mg/L solution of glufosinate ammonium (Bayer CropScience Limited) to select for transgenic plants.

Complementation of *cts-1* with bacterial artificial chromosome clones was as described in Footitt et al. (2002).

Genetic Consequences of *cts* Mutation

Reciprocal crosses were performed between plants heterozygous for the *cts-1* and *cts-2* mutations and (1) wild-type plants or (2) homozygous mutant plants. Plants heterozygous for *cts* alleles were also allowed to self-fertilize. Mature siliques were collected and dried for 1 week prior to sterilization and plating on B5 agarose. Seeds were stratified at 4°C in the dark for 2 d and then transferred to germination conditions, as indicated in Footitt et al. (2006). *cts-1*/*cts-1* and *cts-2*/*cts-2* offspring were scored by failure to germinate under these conditions. Wild-type and heterozygous plants were scored by PCR. Genomic DNA was prepared from seedlings by the method of Edwards et al. (1991), modified for tissue disruption using a Tissue-Lyser (Qiagen Ltd.). PCR reactions contained 1× PCR buffer, 1 unit *Taq* polymerase (Promega), 1.5 mM $MgCl_2$, 0.2 μM dNTPs, 10 pmol primers (see below). For Ws2/*cts-2* progeny, cycle conditions were 94°C, 3 min; 35 cycles of 94°C, 30 s; 55°C, 45 s; 72°C, 3 min; and a final extension of 10 min at 72°C; primers were: DSF1 (5′-TCTAGCTAAGTGGTTGTTGTTGTTAC-3′), DSR2 (5′-CATAGAATGCTATGCTTTCCGAATGAGTC-3′), and JL-202 T-DNA LB primer (5′-CATTTTATAATAACGCTGCGGACATCT-3′). For L*er*/*cts-1* progeny, cycle conditions were 94°C, 3 min; 32 cycles of 94°C, 30 s; 45°C, 45 s; 72°C, 2 min; and a final extension of 10 min at 72°C; primers were cts-cds-forward-04 (5′-GAGATCTTCTATGTGCCGCAACG-3′), cts-cds-reverse-05 (5′-CTTTTCACTGAATCAATTTCAGCATCC-3′), and ChrIVRBF (5′-CCTTCTTTCTTCTCTTCCCCATTTGGTC-3′). Transmission efficiencies were calculated as outlined in Howden et al. (1998) and the predicted frequency of *cts* mutants in self-progeny of +/*cts* heterozygotes was calculated from transmission ratios, as described in Park et al. (1998).

Pollen Tube Growth in Vivo

Pollen tube growth in mutant and wild-type pistils was determined in self- and cross-pollinated pistils. Twenty-four hours after pollination, pistils were removed and stained in aniline blue decolorized with activated charcoal (Muschietti et al., 1994). Images were recorded using a Zeiss Axiophot microscope (Karl Zeiss Ltd), a Leica DFC300FX digital camera, and IM50 Image Manager software (Leica Microsystems).

Pollen Germination and Tube Growth in Vitro

For each genotype, pollen from two flowers was cultured in suspended drops in either control medium [18% (w/v) PEG-3550, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$, 0.015% (w/v) boric acid, pH 6.5] or Suc medium (control medium with 16% [w/v] PEG-3550 and 2% [w/v] Suc). Control and Suc medium were isoosmotic as tested using a vapor pressure osmometer (Wescor). Pollen was incubated in a humid chamber for 16 h in hanging drops on microscope slides. Germination was scored by microscopic examination. Tubes of germinated pollen grains were visualized with a Zeiss Axiovert 135 inverted microscope (Karl Zeiss Ltd), and measured using QWIN image acquisition software (Leica Microsystems). The effect of GABA and GHB on pollen tube growth in vitro was determined as described in Palanivelu et al. (2003).

Statistical Treatments

ANOVA was used to analyze data comprising the percentage fertility, pistil length, percentage pollen germination, and pollen tube length. From the ANOVAs, the appropriate LSDs at the 5% level of significance were used to compare means. A logit transformation was required for the percentage data, but no transformations to other data were required for these analyses.

Localization of Peroxisomes in Floral Tissue Using Confocal Microscopy

A line containing a *35S::GFP-MFP2* fusion protein that is targeted to the peroxisome (Cutler et al., 2000) was used. Flowers of the indicated developmental stage were dissected and mounted in water and enhanced GFP fluorescence visualized on a Zeiss LSM 510 inverted confocal microscope equipped with 10×, 40×, and 63× oil immersion objectives. Excitation was with an argon laser at 488 nm and fluorescence detection using a 505- to 530-nm band-pass filter. Postacquisition image processing was done using LSM 5 browser software (Zeiss) and the Adobe Photoshop suite of programs.

Measurement of GABA and GHB

Plants were grown as described in "Plant Material." Flowers (stage 13) were removed from primary inflorescences at the same time each day, over a 5-d period, and frozen in liquid nitrogen. Floral tissue was freeze dried prior to extraction and derivatization and metabolite content was determined by gas chromatography-mass spectrometry exactly as detailed in Roessner-Tunali et al. (2003), with the exception that retention time standards used were as described in Fait et al. (2006). In addition, GHB was added to the compounds that could be detected by this protocol by running an aliquot of chemically pure GHB purchased from Sigma-Aldrich.

Vegetative Phenotype

Plants of *cts-1* and L*er* were harvested commencing 10 d following transfer to soil and at 5-d intervals until siliques began to shatter. At each harvest, 10 plants were analyzed for rosette and cauline leaf number, area, and dry weight. Silique number and dry weight were also determined, as was the dry weight of the remainder of the aerial plant parts (stem and flowers). Dry weights were determined after 24 h at 90°C. Leaf areas were determined by analysis of leaf images using a Gel Doc 2000 with Quantity One software (Bio-Rad). All data are presented as the mean ± SE. Cuticle integrity was tested in 7-d-old seedlings, as described in Tanaka et al. (2004). The cuticles of *cts* seedlings were indistinguishable from those of wild types (data not shown).

Photosynthesis and Chlorophyll Content

Chlorophyll was extracted from single rosette leaves of known fresh weight with 80% acetone. Chlorophyll content was determined spectrophotometrically after Hendry and Price (1993). Photosynthesis was measured by infrared gas analysis at a light intensity of 400 μmol m^{-2} s^{-1} at 20°C after Dutilleul et al. (2003).

Supplemental Data

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

Supplemental Figure S1. Vegetative phenotype of *cts* mutants.

Supplemental Table S1. The observed and predicted frequency of *cts* mutants in self-progeny of +/*cts* heterozygotes.

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