Running head: peroxisomal ABC transporter and fertility

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The COMATOSE ABC transporter is required for full fertility in Arabidopsis.

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ABSTRACT

COMATOSE (*CTS*) encodes a peroxisomal ABC transporter required not only for β-oxidation of storage lipid during germination and establishment but also for biosynthesis of jasmonic acid (JA) and conversion of indole butyric acid (IBA) to indole acetic acid (IAA). *cts* mutants exhibited reduced fertilisation which was rescued by genetic complementation but not by exogenous application of JA or IAA. Reduced fertilisation was also observed in thiolase (*kat2-1*) and peroxisomal acyl-CoA 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. No differences between mutant and wild-type pollen were observed in the presence of sucrose. Aniline blue staining of pollinated pistils demonstrated that pollen tube growth was affected only when both parents bore the *cts* mutation, indicating that the 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 GABA levels were reduced in flowers of *cts* mutants, they were unchanged in *kat2-1*, suggesting that alterations in GABA 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 and suggest that this pathway could play a role in the mobilisation of lipid in both pollen and female tissues.

INTRODUCTION

In oilseed plants, fatty acids are stored in the seed as triacylglycerol (TAG), which is metabolised 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. The core reactions of the pathway are catalysed 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 (reviewed in 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. Since 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 *PED3* and *AtPXA1*) encodes a peroxisomal ABC transporter which was identified in a genetic screen for positive regulators of germination (Russell et al., 2000). *cts-1* mutant seed cannot germinate in the absence of classical dormancy-breaking treatments and do not establish in the absence of an exogenous energy source owing to their inability to mobilise storage lipid 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 (2,4-DB) and indole butyric acid (IBA) (Hayashi et al., 2002; Zolman et al., 2001). These compounds are converted by one round of β-oxidation to the bioactive auxins, 2,4-dichlorophenoxyacetic acid (2,4-D) and indole acetic acid (IAA), respectively, 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 the 3-ketoacyl-CoA thiolase encoded by *KAT2* or the acyl CoA oxidases, *ACX1* and *ACX5* (Cruz Castillo et al., 2004; Pinfield-Wells et al., 2005; Afitlhile et al., 2006; Li 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 (Stintzi and Browse, 2000; Sanders et al., 2000). Unlike many JA biosynthetic and signalling mutants, however, *cts* mutants are male-fertile. It is probable that the low residual levels of JA in *cts* mutants are sufficient for fertility, since 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 role(s) of this transporter during post-germinative growth, to determine what impact loss of *CTS* function has on vegetative and reproductive processes.

Fertilisation in flowering plants is a multi-step process that requires the delivery of the pollen sperm cells to the ovules which are located deep within the flower (Johnson and Preuss, 2002). Lipid metabolism and signalling potentially play important roles in several stages of fertilisation, starting with production and release of viable pollen (Stintzi and Browse, 2000; Sanders et al., 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 the 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 organisation 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 polarised, tip-growing structures: the pollen tube and the radicle, respectively. Both structures contain abundant pre-synthesised 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 lipid takes the form of triacylglycerols, which are stored together with phospholipids and oleosins in oil bodies (Kuang and Musgrave, 1996; Kim et al., 2002). During pollen formation, oleosins and TAG are synthesised in the abundant ER of tapetosomes and numerous oil droplets are produced in a manner apparently identical to that of seeds (Hsieh and Huang, 2005). Ultrastuctural 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, *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, oil bodies disappear following pollen germination (Rodriguez-Garcia et al., 2003) and accordingly, a putative TAG lipase (*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 mobilisation 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). Since 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 and tobacco (Lord 2000; Lord and Russell, 2002; Lord, 2003). 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-amino butyric 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 post-germinative phenotype of *cts* mutants, with particular attention to fertility. We present data demonstrating that fertilisation 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 fertilisation is the provision of energy for pollen tube germination and growth via mobilisation of TAG in both male and possibly also female tissues.

RESULTS

Post-germinative phenotype of cts mutants

Following mechanical rupture of the testa and seedling establishment in the presence of exogenous sucrose, *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, L*er*, but these parameters were much less affected in *cts-2* mutants, which more closely resembled the wild-type, Ws2 (fig. S1). Cauline leaf number and area were also reduced in *cts-1* (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 1B,C). This ratio was restored in *cts-1* plants expressing the *CTS* open reading frame 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 WT 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. Whilst painting buds with JA did not affect filament extension (data not shown), this parameter was enhanced by spraying with the synthetic auxin analogue, 1-naphthaleneacetic acid NAA, such that treated mutants resembled the WT (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).

Fertilisation 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 WT (fig. 2B,C). Shorter siliques were associated with reduced fertilisation of ovules and also increased abortion of embryos in mutant alleles (fig. 3A-C). The percentage of fertilised 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 fertilisation were restored by genetic complementation (fig. 3A). In order to test whether the fertility defects were specific to *cts* mutants, or reflected a more general defect in β-oxidation, fertilisation was assessed in the single *kat2-1* and the double *lacs6-1, lacs7-1* mutants which are deficient in 3-ketoacyl-CoA thiolase and two peroxisomal long chain acyl CoA synthetases, respectively (Germain et al., 2001; Fulda et al., 2004). The percentages of fertilised ovules and aborted seeds in *kat2-1* were similar to those in *cts* alleles, whilst the reduction in fertilisation 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 on to 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. Jasmonic acid 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 fertilisation following spraying of flower buds with 10 μM NAA.

cts pollen performs less well than WT pollen

Reduced fertility could be due to defects in either sporophytic tissue or in gametophytic tissue. To test for gametophytic effects of the *cts* mutation, plants

heterozygous for *cts-1* or *cts-2* were allowed to self-fertilise and the genotypes of progeny were deduced by germination assays and allele-specific PCR. A significant deviation from the Mendelian ratio of 1:2:1 (WT : +/*cts* : *cts*/*cts*) was observed (Table IA), 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 which 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 IB). 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 IC). 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 (Table ID).

Pollen tube growth is impaired in cts mutants

In *cts* mutants, unfertilised ovules appeared to predominate towards 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 on to 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 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 the same length in both wild-types and mutants (fig. 4B).

Peroxisomes are abundant in many floral tissues

Since aniline blue staining indicated a role for CTS in both male and female tissues, we investigated the abundance of peroxisomes- the organelles which house the β-oxidation pathway- in different floral tissues. Peroxisomes were visualised by confocal scanning laser 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. 5A, B and 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. 5D, E). Peroxisomes were also present within the funiculus and fertilised ovules (fig. 5F).

GABA metabolism in flowers of cts and kat2-1 mutants

Since γ-amino butyric acid (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 *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 0-1 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 sucrose.*

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 iso-osmotic media containing 18 % PEG or 16 % PEG plus 2 % sucrose. The percentage of WT 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 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 sucrose 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. 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 sucrose (fig. 7B). In sucrose media, pollen tubes of the β-oxidation mutants, *cts-1*, *cts-2*, *kat2-1* and the double *lacs6-1,lacs7-1* mutant, were of similar length to those of the respective wild-types. However, the 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 the 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).

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* (fig. S1). This is in 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 multifunctional protein, 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 which 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 signalling is perturbed, for example: the filaments of the auxin response transcription factor mutants, *arf6-2* and *arf8-3* were shorter than those of WT 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, though it is possible that exogenously-applied auxin bypasses a requirement for CTS unrelated to auxin metabolism.

Fertilisation is impaired in β-oxidation mutants

A noticeable feature of the *cts* mutants was the production of shorter siliques (fig. 2), a phenotype which is often indicative of reduced fertilisation, as, for example, in the JA biosynthetic mutant, *opr3* (Stintzi and Browse, 2000) and mutants lacking the VGD pectin methylesterase, which is required for pollen tube growth (Jiang et al., 2005). Dissection of siliques revealed that fertilisation was reduced in both *cts-1* and *cts-2*, with a more marked effect in the latter allele (fig. 3A, B). Fertilisation was also compromised in *kat2-1* and, to a lesser extent, in the double *lacs6-1*,*lacs7-1* mutant (fig. 3B), implying a general role for β-oxidation in fertilisation. Although *cts-1* was complemented by a *CTS* promoter-ORF construct (fig. 3A), the *cts* mutants were not rescued by exogenous application of JA, or its precursors, 18:3 and OPDA, demonstrating that the fertilisation phenotype does not result from JA deficiency. Application of auxin also failed to rescue the *cts* fertilisation 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 which is handled by additional or alternative acyl activating enzymes.

Pollen tube germination and growth are impaired in cts mutants

Whilst *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 (Honys and Twell, 2003, 2004; Becker et al., 2003; Wellmer et al., 2004; Yu et al., 2005). Thus CTS could play a role in either or both sporophytic or gametophytic tissues. A 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 a greater reduction in fertilisation 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 fertilisation.

In accordance with a role for *CTS* in pollen, aniline blue staining revealed that *in vivo* pollen tube growth was impaired when the *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 in 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 which 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 visualised in petals, sepals, funiculus and ovules but were not observed in pollen of *35S::MFP-GFP* 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 ECFP in lily (Prado et al., 2004) and the *ACX1* promoter to drive expression of a peroxisomal EYFP 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 fertilisation, 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 fertilisation in *cts* mutants. Pollen germination in the absence of exogenous sucrose 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 sucrose was included in the medium (fig. 7). These findings are consistent with the suggestion that

catabolism of stored lipid 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; Scholz-Starke et al., 2003; Schneidereit et al., 2003, 2005; 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 sucrose 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 sucrose 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 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 RT-PCR studies also demonstrated lack of expression of malate synthase in floral tissue, whereas 3-ketoacyl thiolase (*PED1*/*KAT2*) is highly expressed (Charlton et al., 2005). In contrast, peroxisomal citrate synthase (*CSY2* and *CSY3*) transcripts are expressed throughout the flower (Zimmerman et al., 2004), suggesting that lipid can be used as a source of carbon for respiration, as has been proposed for germinating sunflower 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, since 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 metabolise 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 lipid. Moreover, the fact that the presence of CTS in either the male or female tissues is sufficient for full fertility suggests that this transporter fulfils 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 characterised (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). Since 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.

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, whilst there may be a minor contribution of *CTS* to GABA metabolism, any alteration in GABA content is unlikely to account for the reduced fertility which 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, since 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 post-translational 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 since 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.

CONCLUSIONS

The CTS ABC transporter plays a key role in regulating import of substrates into the peroxisome for β-oxidation. Whilst *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 of JA and IAA and catabolism of GABA, but did not find evidence that any of these functions underpins the fertilisation phenotype of *cts* mutants. Although we cannot rule out a role for CTS (and β-oxidation) in processing an as-yet unidentified signalling molecule required for efficient fertilisation, our data are consistent with the hypothesis that CTS contributes to fertilisation via the provision of energy and possibly carbon skeletons for the actively-growing pollen tube.

MATERIALS AND METHODS

Plant material

The 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

After-ripened *Arabidopsis thaliana* seeds of the mutants, *cts-1, cts-2,* and their respective wild types (WTs; L*er* and Ws2) were germinated as described in Footitt *et* al., (2006). After seven days, 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 (RH)/ 8 h dark at 18 °C and 80 % RH). During the light phase the incident photosynthetically active radiation (PAR) was 150–175 μmoles m^{-2} sec^{-1} at soil level. The position of plant trays was rotated to minimise light effects.

Floral and fertilisation 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 fertilisation and abortion of ovules in siliques derived from the primary inflorescence was determined at 5, 10 and 15 days after flowering (DAF). Unfertilised ovules appear white while aborted ovules are shrunken and dark in colour. Silique size was measured at increasing DAF.

The ability of jasmonic acid (2.0 mM) (Sigma, Poole, Dorset, UK) and its precursors 3-oxo-2-(2'-Z-pentenyl)-cyclopentane-1-octanoic acid (OPDA; 3.4 mM) (Larodan AB, Sweden), and α-linolenic acid (3.3 mM) (Nu-Chek Prep Inc, USA) to rescue floral and fertilisation 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 (1-naphthaleneacetic acid; NAA, and indole acetic acid; 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). Firstly, annealed oligonucleotides (5'-CTA GAG GAT CCG CAT G-3' and 5'- CGG ATC CT-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, Hitchin Hertfordshire, UK)] 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 bp 2638 – 1445 upstream of the ATG was amplified with primers CTS ProFW2 (5'-GAGTACTTGGAAGAAGGCGGTGA-3') and CTS ProRV9 (5'-ATTGTACACCGCATGATTGAAGCACA-3') and ligated into a blunted *Apa* I site of pBluescript® II SK- (Stratagene, La Jolla, USA) to generate the plasmid, pSKPro5'. A promoter fragment corresponding to bp 1507 upstream and 38 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 *Sac* II site) and ligated into the *Sma* I site of pBluescript® II SK-, to yield pSKPro3'. The open reading frame of *CTS* was then amplified in two parts using cloned cDNA as template. The 5' fragment amplified by primer CTS5'FW (5'-GG**C**CG**C**GGTCTTGTAGCGTCAAGACGG A-3' bold type indicates silent nucleotide changes to introduce *Sac* II site) and CTS5'RV (5'-GCCTTTGAATTAGTAGCAGATTCC-3') and cloned in pCR-Blunt II Topo (Invitrogen, Paisley, UK), to yield pCRBlunt ORF5', and the 3' fragment amplified by primer CTS3'FW2 (5'-GATCGGCAAAATGATGCGATGGT-3') and CTS3'RVwStop (5'-**CCCGGG**TCACTCTGTTGTCTGTTCGATCGA-3'; bold type indicates introduced *Sma* I site) was restricted with *Pst* I and ligated in the *Pst* I/*Eco*

RV sites of pBluescript® II SK-, to generate pSK ORF3'. The promoter and the 5' portion of the *CTS* ORF were assembled in a three-way ligation between pSK Pro5' restricted with *Bbs* I/*Pst* I, pSK Pro3' 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, Hauxton, Cambridge, UK) to select for transgenic plants.

Complementation of *cts-1* with BAC 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 (a) wild type plants or (b) homozygous mutant plants. Plants heterozygous for *cts* alleles were also allowed to self-fertilise. Mature siliques were collected and dried for one week, prior to sterilisation and plating on B5 agarose. Seeds were stratified at 4 ºC 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 TissueLyser (Qiagen Ltd., Crawley, West Sussex, UK). PCR reactions contained 1 x 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 ºC, 3 min; 35 cycles of (94 ºC 30 sec, 55 ºC 45 sec, 72 ºC, 3 min) and a final extension of 10 min at 72 ºC; primers were: DSF1 (5'-TCTAGCTAAGTGGTTGTTGTTGTTGTTAC-3'), DSR2 (5'-CATAGAATGCTATGCTTTCCGAATGAGTC-3') and JL-202 T-DNA LB primer (5'-CATTTTATAATAACGCTGCGGACATCT-3'). For Ler/*cts-1* progeny, cycle conditions were 94 ºC, 3 min; 32 cycles of (94 ºC 30 sec, 45 ºC 45 sec, 72 ºC, 2 min) and a final extension of 10 min at 72 ºC; 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 WT pistils was determined in self- and cross-pollinated pistils. 24 h after pollination, pistils were removed and stained in aniline blue decolourised with activated charcoal (Muschietti *et al*., 1994). Images were recorded using a Zeiss Axiophot microscope (Karl Zeiss Ltd, Welwyn Garden City, UK), Leica DFC300FX digital camera and IM50 Image Manager software (Leica Microsystems UK, Ltd, Milton Keynes, Bucks, UK).

***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 sucrose medium [control medium with 16 % (w/v) PEG-3550 and 2 % (w/v) sucrose]. Control and sucrose medium were iso-osmotic as tested using a vapour pressure osmometer (Wescor, Logan, Utah, USA). 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 visualised with a Zeiss Axiovert 135 inverted microscope (Karl Zeiss Ltd, Welwyn Garden City, UK), and measured using QWIN image acquisition software (Leica Microsystems UK, Ltd, Milton Keynes, Bucks, UK). The effect of GABA and GHB on pollen tube growth *in vitro* was determined as described in Palanivelu et al., 2003.

Localisation of peroxisomes in floral tissue using confocal microscopy

A line containing a 35S GFP-MFP2 fusion protein which is targeted to the peroxisome (Cutler et al., 2000) was used. Flowers of the indicated developmental stage were dissected and mounted in water and eGFP fluorescence visualised on a Zeiss LSM 510 inverted confocal microscope equipped with 10x, 40x and 63x oil

immersion objectives. Excitation was with an argon laser at 488 nm and fluorescence detection using a 505-530 nm band pass filter. Post-acquisition image processing was done using the LSM 5 browser software (Zeiss) and the Adobe Photoshop suite of programmes.

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 five day period and frozen in liquid nitrogen. Floral tissue was freeze-dried prior to extraction and derivatisation and metabolite contents were determined by GC-MS 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, GBH was added to the compounds that could be detected by this protocol by running an aliquot of chemically pure GBH purchased from Sigma Aldrich (Munich, Germany).

SUPPLEMENTAL MATERIAL

Materials and methods

Vegetative phenotype

Plants of *cts-1* and L*er* were harvested commencing ten days following transfer to soil and at five day intervals until siliques began to shatter. At each harvest ten plants were analysed 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 & 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 (Biorad). All data are presented as the mean ± SE. Cuticle integrity was tested in seven day old seedlings as described in Tanaka *et al.* (2005). 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 μmoles m^{-2} s^{-1} at 20 °C after Dutilleul *et al.* (2003).

Fig. S1. Vegetative phenotype of *cts* mutants

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FIGURE LEGENDS

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 thaliana*, accession Landsberg *erecta* (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 1-naphthaleneacetic acid (NAA).

Figure 2. Silique production in *cts* mutants and respective wild types.

A. Total silique production. B. Total silique dry weight. C. Silique length. Closed circles, L*er*; open circles, *cts-1*. Closed triangles, Ws2; open triangles, *cts-2*. Values are means ± SE (n=10-17).

Figure 3. Fertilisation in *cts* mutants.

A. Percentage fertilised 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 d after flowering. Values are means ± SE (n = 10-15 siliques). B. Percentage fertilised 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). C. Dissected siliques (harvested 10 d after flowering) showing unfertilised ovules (white arrowheads) and aborted embryos (red arrowheads) in *cts* mutants.

Figure 4. Pollen tube growth in *cts* mutants and respective wild types.

A. Pollen tube growth in mutant and WT 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).

Figure 5. Peroxisomes in reproductive tissues of Arabidopsis.

Peroxisomes were visualised by confocal laser scanning microscopy of plants expressing peroxisomally-targeted EGFP. A. Stage 12 flower showing GFP expression in petal, stamens (anther and filament) and pistil. Scale bar 50μm. 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 sucrose-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 fertilised ovule and funiculus from a stage 16 flower. Scale bar 10 μm.

Figure 6. GABA and GABA shunt metabolite content in flowers.

Panel A: Proposed scheme for γ-amino butyric acid (GABA) metabolism in Arabidopsis flowers. GABA is synthesised in the cytosol from glutamate by glutamate decarboxylase (GAD). The first step in GABA catabolism is catalysed by mitochondrial GABA transaminase (GABA-T). In Arabidopsis flowers, this enzyme is encoded by *POP2*, and utilises pyruvate (pyr) to yield alanine (ala) and succinic semialdehyde (SSA). Mutation of *POP2* results in elevated GABA. In mitochondria, SSA undergoes oxidation to succinate, catalysed 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 γ-hydroxybutyrate (GHB). It is possible that GHB, a short chain hydroxy fatty acid, is metabolised 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.

Panel B: content of γ-amino butyric acid (GABA), γ-hydroxybutyrate (GHB) and succinate in Arabidopsis flowers. Values are means ± SE (n = 4). A T-test was performed comparing every mutant and its respective WT (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 WTs.

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 % sucrose). Values are means ± SE of three independent slides (n = ca 800 grains/slide). B. Pollen tube growth in different genotypes: L*er* (WT), *cts-1* and *cts-1* transformed with BAC clone 159N1 (*cts-1*::*CTS*); Ws2 (WT), *cts-2*, *kat2-1* and *lacs6-1 lacs7-1*. Pollen was germinated *in vitro* in the presence or absence of a carbon source (2 % sucrose) and pollen tube lengths of germinated pollen determined after 18 h. Values are means ± SE (n=50). Data presented in A and B are representative of several independent experiments

Figure S1. Vegetative phenotype of *cts* mutants: leaves.

Values are means ± SE for 10 plants.

Table I. Genetic transmission of *cts* alleles

A. Observed frequency of different genotypes in self progeny of +/*cts* heterozygotes. B. Offspring ratios 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). C. Transmission efficiency (TE) of *cts* alleles, calculated from data in B**.** TE represents the fraction of mutant gametes that successfully transmit the mutation (Howden *et al*., 1998) and is defined as: TE (%) = (# mutants)/(#WT plants) x 100. D. The predicted frequency of *cts* mutants in self progeny of +/*cts* heterozygotes, calculated from transmission ratios shown in C, as described in Park *et al*., 1998.

A

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

B

♀ **parent**	♂ **parent**	**# offspring**			**Probability**
		+/+	***cts-1*/+**	***cts-1*/*cts-1***	
+/+	*cts-1*/+	97	21		P < 0.001
cts-1/+	+/+	49	46		P > 0.05
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
cts-2/+	+/+	60	68		P > 0.05
cts-2/+	*cts-2*/*cts-2*		72	56	P > 0.05
cts-2/*cts-2*	*cts-2*/+		151	15	P < 0.001

C

Cross	**+/+**	**+/*cts***	***% cts***	**TE (%)**
+/+ x +/*cts-1 (male transmission)*	97	21	17.8 %	**21.6**
+/*cts-1* x +/+ *(female transmission)*	49	46	48.4 %	**93.9**
+/+ x +/*cts-2 (male transmission)*	107	11	9.3 %	**10.3**
+/*cts-2* x +/+ *(female transmission)*	60	68	53.1 %	**111.3**

D

		(+/*cts*) + (*cts*/*cts*)	**+/+**
self-progeny of +/*cts-1*	Predicted	57.6 %	42.4 %
	Observed	88 (41.7 %)	123 (58.3 %)
self-progeny of +/*cts-2*	Predicted	57.6 %	42.5 %
	Observed	123 (56.4 %)	95 (43.6 %)