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RESEARCH PAPER

Analysis of the role of *COMATOSE* and peroxisomal beta-oxidation in the determination of germination potential in *Arabidopsis*

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Abstract

Comparative physiological analysis of mutant *Arabidopsis* seeds under defined environmental conditions was used to analyse the relative contributions of components of peroxisomal beta-oxidation in the control of seed germination potential. The *COMATOSE* (*CTS*) and *KAT2* loci were shown to play essential roles in regulating germination and establishment potentials, whereas *LACS6* and *LACS7* loci only influenced establishment following germination. The viability and desiccation tolerance of three different mutant alleles of *CTS* were shown to be intermediate between that of dormant and non-dormant wild-type seeds. Analysis of *ttg-1 cts-1* double mutant seeds demonstrated that the *cts* lesion did not influence after-ripening capacity. These data demonstrate the importance of peroxisomal beta-oxidation in the control of germination potential, but suggest that breakdown of stored lipid is not an important prerequisite for germination. A function is suggested for CTS following after-ripening within pathways related to the progression of germination prior to radicle emergence.

Key words: *Arabidopsis*, beta-oxidation, COMATOSE, gene expression, germination, peroxisome, physiological genetics.

Introduction

Processes associated with successful seed germination are coupled to three temporal phases (Bewley and Black, 1994). Phase one (the imbibition phase) commences with imbibition of water by the dry seed. Following the initiation of imbibition, those biochemical programmes connected with the resumption of cellular processes (e.g. DNA repair, translation, re-initiation of metabolism) are associated with the second (germination) phase (Bewley, 1997). Biochemical processes within the second phase determine the developmental fate of the seed. Mature dry seeds undergo a process of after-ripening, through which primary dormancy is lost. Following the initiation of imbibition, seeds that are not after-ripened will remain dormant within phase two, whilst seeds that have after-ripened will proceed to phase three. Phase three starts with radicle emergence, which precedes post-germination seedling establishment. Radicle emergence, therefore, marks the end of the germination phase and the onset of seedling growth leading to establishment. Environmental and genetic cues are integrated by imbibed seeds and determine the developmental pathway (dormancy or germination) that is initiated during phase two of germination. After-ripening and chilling are presumed to stimulate germination by increasing sensitivity to factors that increase germination potential (Hilhorst and Karssen, 1992).

The biochemical processes controlling after-ripening, dormancy maintenance, and germination are poorly understood. In *Arabidopsis*, translation of RNA is essential for germination and seedling establishment to proceed,

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whereas gene transcription producing novel RNA is only required for establishment (Rajjou *et al.*, 2004). This suggests that all RNA–protein molecules required for germination are expressed during embryo maturation and are therefore present in the dry mature seed.

In recent years, genetic studies in *Arabidopsis* have defined a large number of genes that influence germination and establishment. These can broadly be divided into three functional groups: those that influence seed structure (particularly of the maternally derived testa), those that control development (e.g. hormone metabolism or transduction), and those that encode proteins associated with metabolic pathways important for seedling growth and survival. The developmental response observed in imbibed seeds is the result of the combined action of these (and other as yet undiscovered) genes. Phytohormones have a controlling influence in determining germination potential. The relative effects of either abscisic acid (ABA) or gibberellin are particularly important in defining the dormancy to germination transition (Holdsworth *et al.*, 1999; Koornneef *et al.*, 2002). The effect of ABA synthesis and signal transduction has been associated principally with the establishment and maintenance of dormancy (i.e. reduced germination potential) (Koornneef *et al.*, 1982, 1984; Finkelstein and Somerville, 1990; Hugouvieux *et al.*, 2001; Kushiro *et al.*, 2004), whereas gibberellin biosynthesis activates germination and germination-associated gene expression profiles (Ogawa *et al.*, 2003). Members of the DELLA-domain protein family, in particular RGL2, act as negative regulators of gibberellin action by repressing germination through inhibition of the gibberellin signal transduction pathway (Lee *et al.*, 2002). In addition, ethylene and brassinosteroids have also been shown to influence germination potential positively (Kepczynski and Kepczynska, 1997; Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Steber and McCourt, 2001; Brady and McCourt, 2003).

Forward and reverse genetic screens have identified several *Arabidopsis* loci which encode proteins implicating specific biochemical pathways in the control of both embryo germination potential and seedling establishment. There is also evidence of an important and differential role of the endosperm in ABA responsiveness of metabolism during seedling establishment (Penfield *et al.*, 2004). Analysis of mutant plants lacking key components of the glyoxylate cycle (malate synthase, *mls*, and isocitrate lyase, *icl-2*) demonstrated that this pathway is not essential for seedling growth, but is used to aid establishment under suboptimal growth conditions (Eastmond and Graham, 2001; Cornah *et al.*, 2004). Beta-oxidation and downstream metabolic pathways in the glyoxysome of the embryo have been shown to be the major routes through which stored fatty acids are utilized to drive seedling establishment in *Arabidopsis* (Germain *et al.*, 2001; Cornah *et al.*, 2004; Penfield *et al.*, 2005; Pracharoenwattana *et al.*, 2005; Baker *et al.*, 2006). Loss of function of components of beta-oxidation through mutation leads to an inability of seedlings to establish (i.e. to arrive at photoautotrophic growth via cotyledon development) in the absence of an external sugar supply (Germain *et al.*, 2001; Footitt *et al.*, 2002; Fulda *et al.*, 2004; Pinfield-Wells *et al.*, 2005; Pracharoenwattana *et al.*, 2005). The finding that the *COMATOSE* (*CTS*) locus controls germination potential suggested that beta-oxidation is also essential for successful completion of the germination phase prior to radicle emergence (Footitt *et al.*, 2002). The *CTS* gene encodes a full-length ABC-transporter that is required for the metabolism of very-long-chain fatty acids in the glyoxysome during seedling establishment, most likely by acting as a transporter for these fatty acids or their CoA derivatives (Footitt *et al.*, 2002), and has also been implicated in the transport of other biologically important substrates, including indole butyric acid (Zolman *et al.*, 2001) and 12-oxophytodienoic acid, a jasmonic acid (JA) precursor (Theodoulou *et al.*, 2005, 2006). Following transport, these substrates are subject to beta-oxidation in the peroxisome. Recently the importance of other proteins associated with beta-oxidation (acyl-CoA oxidase, ACX1/2, and citrate synthase, CSY2/3) for germination potential have also been demonstrated (Pinfield-Wells *et al.*, 2005; Pracharoenwattana *et al.*, 2005).

It has been shown previously that mutation of the *CTS* locus results in an inability to complete germination that was previously suggested to imply a 'forever dormant' phenotype (Russell *et al.*, 2000). This assertion was strengthened by the observation that protein profiles in imbibed *cts-1* mutant seeds closely resembled those in dormant seeds (Russell *et al.*, 2000). However, specific experiments would be required to distinguish between a phenotype related to dormancy and one in which germination potential is disrupted. In this paper, a standard set of germination conditions was utilized to analyse the germination potential of available mutant alleles of several important genes associated with beta-oxidation. A comparative physiological and genetic analysis is reported that has made it possible to define more specifically the function of *CTS* and beta-oxidation in the determination of germination potential and subsequent seedling establishment.

Materials and methods

Plant materials

Original *Arabidopsis thaliana* seed lots were obtained from NASC (University of Nottingham, UK) unless otherwise stated. Seeds of the single mutants (Table 1), *cts-1* (L*er*) (Footitt *et al.*, 2002), *cts-2* (Ws2) (Footitt *et al.*, 2002), *pxa1-1* (Col-0) (Zolman *et al.*, 2001), *icl-2* (Col-0) (Eastmond *et al.*, 2000), *mls* (Ws4) (Cornah *et al.*, 2004), *kat2-1* (Ws4) (Germain *et al.*, 2001), and *opr3-1* (Ws2) (Stintzi and Browse, 2000) and double mutants *lacs6-1 lacs7-1* (Ws2) (Fulda *et al.*, 2004), *ttg1-1 cts-1* (L*er*) (previously reported in Footitt *et al.*, 2002), and their respective control wild types (WTs) and ecotype CVI

Table 1. *Description of mutant genes, alleles and ecotypes used in this study*

Protein(s)	Process	AGI code	Mutant allele	Nature of allele	Background	Reference
COMATOSE ABC transporter	Beta-oxidation	At4g39850	*cts-1*	Fast neutron translocation	Landsberg *erecta* (L*er*)	Russell *et al*., 2000
COMATOSE ABC transporter	Beta-oxidation	At4g39850	*cts-2*	T-DNA insertion	Ws2	Footitt *et al*., 2002
COMATOSE ABC transporter	Beta-oxidation	At4g39850	*pxa1-1*	Point mutation	Col-0	Zolman *et al*., 2001
Thiolase	Beta-oxidation	At2g33150	*kat2-1*	T-DNA insertion	Ws4	Germain *et al*., 2001
Very-long-chain acyl-CoA synthetases	Beta-oxidation	At3g05970 At5g27600	*lacs6-1 lacs7-1*	T-DNA insertions	Ws2	Fulda *et al*., 2004
Isocitrate lyase	Glyoxylate cycle	At3g21720	*icl-2*	T-DNA insertion	Col-0	Eastmond *et al*., 2000
Malate synthase	Glyoxylate cycle	At5g03860	*mls*	T-DNA insertion	Ws4	Cornah *et al*., 2004
Oxophytodienoic acid reductase 3	Jasmonate biosynthesis	At2g06050	*opr3-1*	T-DNA insertion	Ws2	Stintzi and Browse, 2000

were produced from 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 150–175 μmol m^{-2} s^{-1} at soil level. Seeds were harvested when plants had ceased flowering and siliques were starting to dehisce. Seeds were cleaned by passing them several times through a sieve (500 μm mesh). They were stored in glassine bags in the dark in a controlled environment incubator at 24 °C. Primary dormancy was therefore removed by dry storage at 24 °C. In the case of CVI, dormancy of dry seeds was maintained by storage at −20 °C in an air-tight container.

Germination and seedling establishment conditions

For all germination analyses, unless otherwise stated, both WT and mutant samples were obtained from plants grown at the same time in the same controlled environment chambers, using seed collected at the same time and stored together for the same time period in a controlled environment incubator. Therefore, as far as possible, previous environmental history and after-ripening time were equivalent for all seed lots tested. Prior to germination, test seeds were surface-sterilized in 5% (v/v) bleach for 5 min, then washed three times in sterile water. For each mutant and ecotype seed lot analysed, seeds were plated onto sterile filter paper or 100 μm nylon mesh supports (Lockertex, UK) on 0.7% (w/v) agarose (type PGP, Park Scientific Ltd.) and incubated at 22 °C under continuous light (150 μmol m^{-2} s^{-1}) for 7 d, at which point germination was scored. Mutants were tested in their response to supplements in the germination media as described. Water agarose was supplemented with combinations of Gamborg's B5 salts (Duchefa, NL) (pH 5.8), 0.5% (w/v) sucrose, and/or 25 mM KNO_3. Germination was scored as radicle emergence, and establishment as the completion of expansion and greening of the cotyledons. In all cases experiments were carried out in (at least) triplicate, using 50–100 seeds per replicate. All germination data are expressed as the mean with standard error of the mean.

Sucrose utilization test

Seeds of the mutants, *cts-2*, *kat2-1*, *lacs6-1 lacs7-1*, and respective ecotypes (Ws2 and 4) were incubated under standard germination conditions as above on Gamborg's B5 salts. Germination was recorded and soluble carbohydrates were extracted in triplicate from 50 seed samples and analysed for sucrose content as described previously (Footitt *et al.*, 2002).

Retention of viability and desiccation tolerance

Retention of viability was tested after increasing periods of incubation of seeds on water agarose under germination conditions. Seeds were subsequently transferred to Gamborg's B5 salts with 0.5% (w/v) sucrose (pH 5.8). The testa/endosperm layers covering the embryo of each seed were gently ruptured with the point of a syringe needle to induce radicle protrusion as a measure of viability. To test the retention of desiccation tolerance in seeds, after increasing periods of incubation under germination conditions, seeds were transferred to sterile, sealed Petri dishes. Seeds were then allowed to reach ambient moisture content by incubation in the dark at room temperature for 7 d before being transferred to Gamborg's B5 salts with 0.5% (w/v) sucrose (pH 5.8) for viability testing. Germination was scored after 7 d.

After-ripening and dormancy breaking

Seeds of the double mutant *ttg1-1 cts-1* were collected in the dormant state from yellowing siliques of the primary bolt. Seeds were stored as described above for increasing time periods. The response of fresh-dormant and progressively after-ripening seeds to 25 mM KNO_3 and exogenous sucrose was tested using standard germination assay conditions.

Stratification tests

Seeds of *cts* alleles and *kat2-1* were placed on agarose plates supplemented with 0.5% (v/v) sucrose and incubated in the dark at 4 °C for increasing periods of time. Plates were then transferred to germination conditions, as described above. Germination was scored 7 d after transfer.

Results

Comparative analysis of mutant germination phenotypes

There are numerous reports in the literature of *Arabidopsis* mutants with altered germination potential; however, different growth/seed storage conditions are frequently employed in different laboratories, often precluding useful comparative analyses. This has been noted previously for a range of species (Cohn, 1996). Different mutant alleles of the *CTS* gene (alternatively named *PED3*/*PXA1*) identified in different laboratories apparently exhibit different germination phenotypes. For example, various *ped3* alleles, and the allele *pxa1-1* are reported to have normal germination potential but require sucrose for establishment, whereas *cts-1* seeds do not complete germination, and require sucrose for both germination and seedling establishment (Zolman

et al., 2001; Footitt *et al.*, 2002; Hayashi *et al.*, 2002). All alleles appear to show 2,4-DB resistance for seedling root growth. Therefore, in this paper, phenotypes of mutant seeds have been compared directly using a series of conditions developed for a standard analysis of germination potential. These conditions included manipulation of factors associated either with environmentally (e.g. light, nitrate) or physiologically (after-ripening status) altered germination potential (Hilhorst and Karssen, 1992). The conditions were used to analyse a range of mutants involved in peroxisomal beta-oxidation, the breakdown of stored lipid and the glyoxylate cycle. Since germination potential is highly ecotype-dependent, the respective WT seeds were also analysed (Alonso-Blanco *et al.*, 2003). Mutant genotypes and respective ecotypes used in this study are listed in Table 1.

An analysis of germination potential of mutant alleles used in this study is shown in Fig. 1. The germination potential of non-stratified, incompletely after-ripened populations of WT seeds differed with respect to ecotype, with the rate of after-ripening amongst populations being influenced by ecotype as reported previously (Alonso-Blanco *et al.*, 2003). Seeds of the ecotype L*er* after-ripened fastest and Ws2 slowest with Col-0 and Ws4 intermediate in response. The germination potential of all ecotypes was increased in the presence of a nitrate source, whereas sucrose did not influence germination potential (Fig. 1A). A range of germination and establishment phenotypes were observed in the different mutant genotypes analysed. After-ripening status did not markedly influence the germination response of the mutants studied (compare A and B in Fig. 1). Under these conditions the glyoxylate cycle mutants *icl-2* and *mls* behaved comparably to their corresponding WTs; however, the three *cts* alleles (*cts-1*, *cts-2*, and *pxa1-1*) and *kat2-1* showed a low germination potential. The effect of dry after-ripening time showed that seeds fell into two groups with respect to their germination potential (Fig. 1B). The *icl-2* and *mls* single mutants exhibited a high germination potential, as did the corresponding WTs. By contrast, the germination potential of the *cts* alleles and *kat2-1*, although slightly higher than partially after-ripened mutant seed populations (Fig. 1A), remained low. Germination of *kat2-1* and *pxa1-1* could be stimulated to some extent by sucrose and Gamborg's B5 salts, but this treatment had little effect on *cts-1* and *cts-2* (Fig. 1B). For those mutants whose after-ripening requirements were not investigated, i.e. *opr3-1* (defective in the production of JA) and the double acyl-CoA synthetase mutant *lacs6-1 lacs7-1*, all after-ripened seeds completed germination (Fig. 1B). The ability of fully after-ripened seeds to establish following germination was examined (Fig. 2). For WTs, all seeds that completed germination successfully established regardless of treatment. This was also the case for *icl-2*, *mls*, and *opr3-1*. Previously the *lacs6-1 lacs7-1* double mutant was shown to require sucrose to complete seedling establishment (Fulda

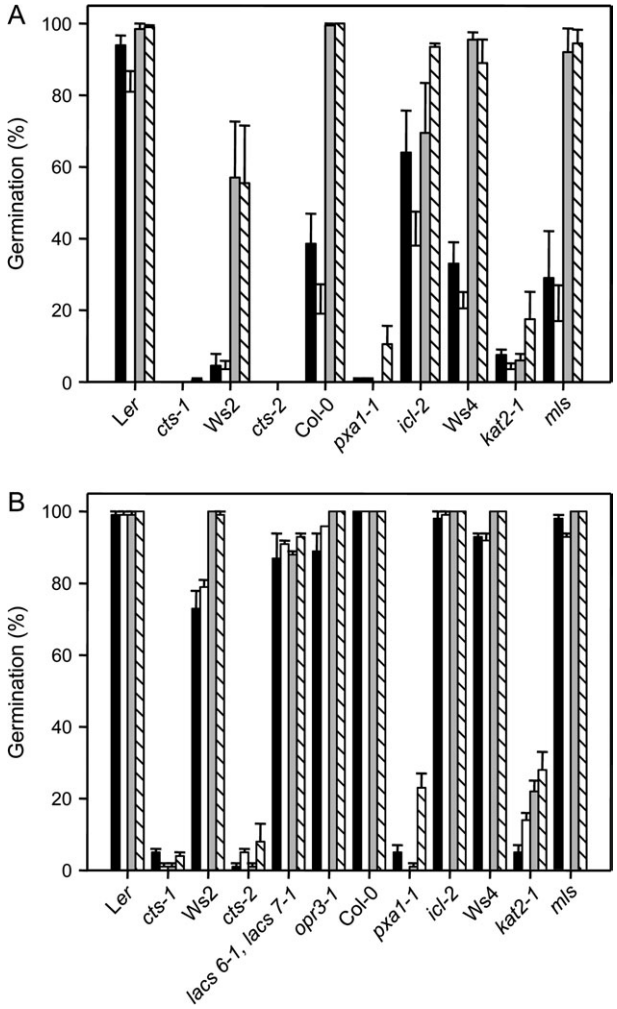

Fig. 1. Germination potential of wild-type (WT) and mutant seeds from two populations showing different degrees of after-ripening. (A) Percentage germination of seeds showing intermediate after-ripening status on four different media. (B) Percentage germination of after-ripened seeds on four different media. Control ecotypes and mutant genotypes are indicated. Black columns, water agarose; open columns, water agarose+0.5% sucrose; grey columns, Gamborg's B5 salts; hatched columns, Gamborg's B5 salts+0.5% sucrose.

et al., 2004). In agreement with this, double mutant seeds germinated on all media, but seedling establishment only occurred in the presence of sucrose (Figs 1, 2). For both *kat2-1* and *cts* alleles, upon the completion of germination, seedling establishment only occurred in the presence of sucrose, as previously reported (Fig. 2) (Germain *et al.*, 2001; Zolman *et al.*, 2001; Footitt *et al.*, 2002; Hayashi *et al.*, 2002). The *opr3-1* mutant contains a T-DNA insertion within the gene encoding 12-oxo-phytodienoic acid (OPDA) reductase, a peroxisomal enzyme which catalyses a key step in the biosynthesis of JA (Schaller *et al.*, 2000; Stintzi and Browse, 2000). Since CTS may transport OPDA (Theodoulou *et al.*, 2005), it was of interest to test whether *opr3-1* exhibited an altered germination phenotype under the controlled conditions

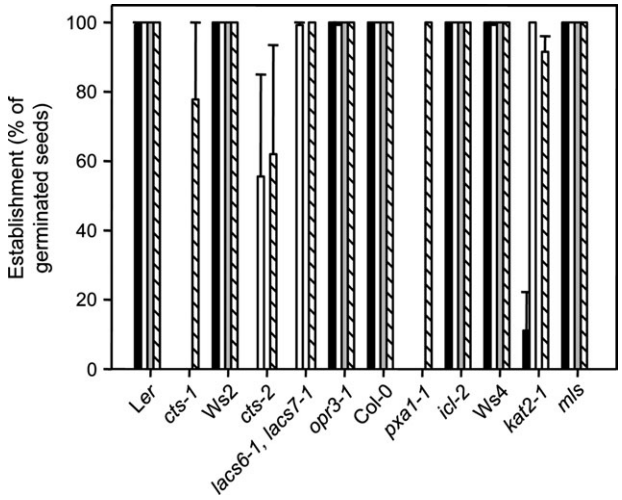

Fig. 2. Establishment of seedlings derived from wild-type and mutant seeds. Establishment as a percentage of germinated after-ripened seeds on four different media (data taken from germinated seedlings from Fig. 1B). Control (WT) ecotypes and mutant genotypes are indicated. Black columns, water agarose; open columns, water agarose+0.5% sucrose; grey columns, Gamborg's B5 salts; hatched columns, Gamborg's B5 salts+0.5% sucrose.

used in the current study. After-ripened seeds of *opr3-1* completed germination and seedlings established on all media tested (Figs 1B, 2).

Previously, sucrose utilization had been shown to differ between WT and *cts* and *kat2-1* mutants during the processes of germination and establishment (Footitt *et al.*, 2002; Pritchard *et al.*, 2002). Comparison of sucrose utilization of *cts-2*, *kat2-1*, and the *lacs6-1 lacs7-1* double mutant with WT demonstrated that all mutants showed a slow decline in sucrose levels, although only WT and *lacs6-1 lacs7-1* double mutant seeds completed germination (Fig. 3). In the *lacs6-1 lacs7-1* double mutant, seed germination was slower than in WT with respective times to 50% germination of 4.5 d versus 1.5 d.

Analysis of the developmental status of cts *mutant seed*

It is well known that dormant seeds retain their viability and desiccation tolerance in the hydrated state (Baskin and Baskin, 1998). In the soil, dormant seeds retain their viability for long periods in the hydrated state, while desiccation tolerance allows both dormant and non-dormant seeds to survive fluctuations in moisture content. The *cts-1* allele was previously proposed to be in a 'forever dormant' state due to the marked reduction in germination potential of stored seeds (Russell *et al.*, 2000; Footitt *et al.*, 2002). To characterize in detail the development of the mutant and, thereby, define more closely the function of the WT CTS protein, the retention of these characteristics in *cts* mutant seeds was tested. Stored *cts* seeds were plated on water agarose and incubated under germination conditions (that would result in the germination of WTs) for the period of

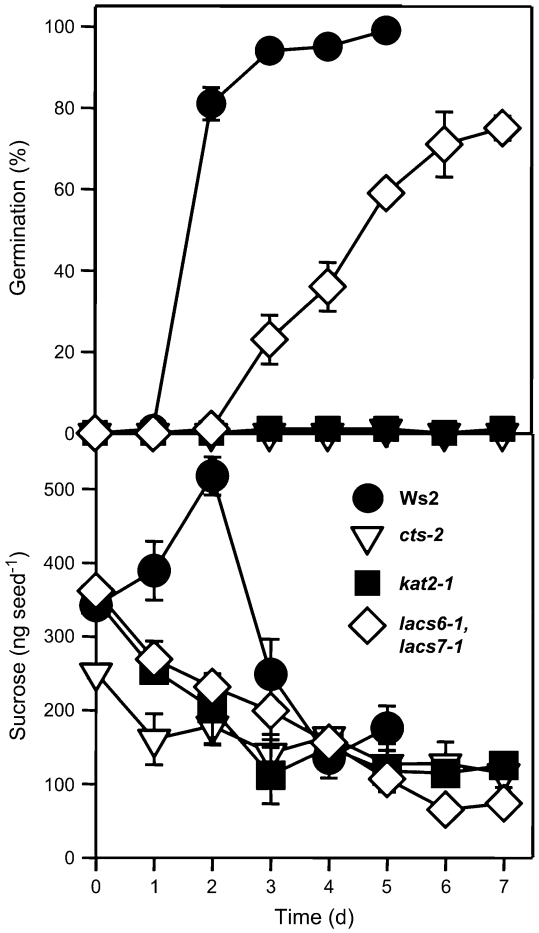

Fig. 3. Sucrose utilization in imbibed seeds of Ws2 and the mutants *cts-2*, *kat2-1*, and *lacs6-1 lacs7-1*. Seeds were incubated on media containing Gamborg's B5 salts for up to 7 d under germination conditions. Seed germination (%) and sucrose content were determined daily. Data for Ws4 were essentially the same as for Ws2 (data not shown).

time indicated and were then induced to complete germination by mechanical rupture of the seed coat and transfer to medium containing Gamborg's B5 salts and 0.5% sucrose. The CVI ecotype, previously shown to have very high levels of dormancy (Alonso-Blanco *et al.*, 2003), was used as an exemplar of dormant seed physiological characteristics. Viability was measured by analysis of germination potential following transfer. Viability of *cts* seeds decreased over time in the hydrated state (Fig. 4). Although this was observed in all three alleles, the loss of viability was most marked for *cts-1*. Dormant CVI seeds retained viability over the duration of the experiment as expected. Desiccation tolerance of seeds in the hydrated state was analysed by carrying out seed drying experiments (Fig. 5). At increasing time periods on water agarose, seeds were removed, dried at ambient temperature for 7 d, then analysed for germination potential following rupture of the external seed structures. Desiccation tolerance was completely lost after 3 d in Col-0, L*er*, and Ws ecotypes, which had already completed radicle emergence before desiccation

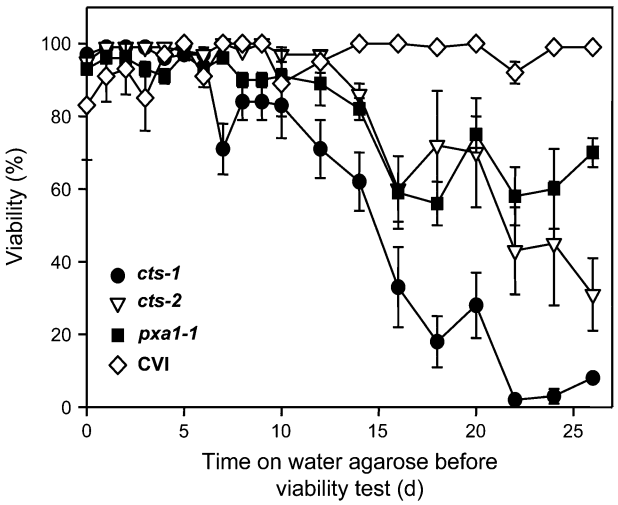

Fig. 4. Retention of viability in seeds of *cts* mutant alleles in comparison to dormant ecotype CVI. After increasing time on water agarose, seed viability was tested by seed coat removal and incubation of seeds on Gamborg's B5 salts+0.5 % sucrose (w/v) for 7 d. CVI germination was recorded after 14 d as this genotype germinates more slowly than L*er*.

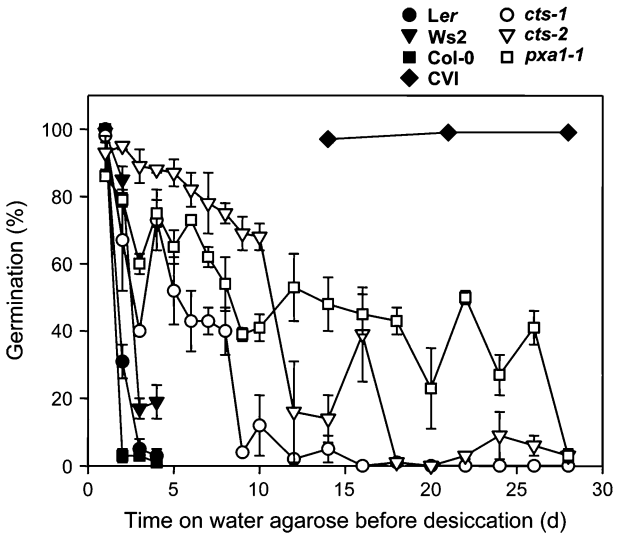

Fig. 5. Desiccation tolerance of stored seeds of *cts* mutant alleles in comparison with respective after-ripened ecotypes and dormant ecotype CVI. After increasing periods on water agarose, seeds were removed and dried at ambient temperature for 7 d, then transferred to Gamborg's B5 salts+0.5% sucrose (w/v). Seed coats were removed and germination measured after 7 d. CVI germination was recorded after 14 d as this genotype germinates more slowly than L*er*.

(Fig. 5). For dormant CVI seeds, desiccation tolerance was retained for the entire experimental period. By contrast, desiccation tolerance was partially retained in the *cts* alleles tested, with retention being greatest in *pxa1-1*.

Data shown in Figs 4 and 5 suggest that *cts* mutant seeds are physiologically distinct from after-ripened WT seed, displaying aspects associated with dormancy (increased desiccation tolerance and long-term viability). To determine whether *cts-1* seeds undergo an after-ripening process, the influence of the *cts-1* allele was analysed in the *ttg1-1* genetic background. Single mutant *ttg1-1* seeds are completely non-dormant, and freshly harvested seed germinate on water agarose on removal from siliques (Debeaujon *et al.*, 2000). The *ttg1-1* phenotype is associated with the maternal testa structures and the physical change to the testa in the *ttg-1* mutant allows entry of external stimuli (including chemicals). Therefore, this mutant characteristic provides an opportunity to investigate the influence of chemicals on CTS function in the embryo, without the need for physical removal of the surrounding structures and disruption of seed integrity. Previous work demonstrated that the *ttg1-1 cts-1* double mutant does not show the reduced dormancy of the *ttg1-1* single mutant, demonstrating that *CTS* is a required function that permits the non-dormant phenotype of *ttg1-1* (Footitt *et al.*, 2002). The germination potential of double mutant seeds on media with or without added nitrate or sucrose was investigated. Nitrogen compounds have been shown to relieve dormancy in many species including *Arabidopsis* (Footitt and Cohn, 2001; Alboresi *et al.*, 2005). Germination potential of *ttg1-1 cts-1* seeds on water agarose was not influenced by time of storage, and double mutant seeds failed to complete germination on this medium regardless of storage time. Mutant seeds incubated on media containing sucrose showed an increase in germination potential that was positively correlated with the duration of dry seed storage (Fig. 6). Seed stored for the greatest period of time completed germination to the greatest percentage at low concentrations of added sucrose. This indicates that the competence to respond to sucrose is dependent on time of storage, suggesting seeds containing the *cts-1* mutation do after-ripen. The addition of nitrate resulted in a further increase in germination potential, but only in the presence of sucrose (Fig. 6B). Moist chilling (stratification) increased the germination potential to >80% in all seed samples in the presence of sucrose with or without nitrate (data not shown). This response to moist chilling in the presence of sucrose was further investigated by stratifying imbibed seeds of *cts* alleles for up to 7 d before transferring to germination conditions. Increasing duration of moist chilling led to an increase in the germination response of all *cts* alleles and the *kat2-1* mutant. This response peaked at 2–3 d of exposure. The germination potential of all *cts* alleles declined thereafter, whereas *kat2-1* germination potential remained constantly high (Fig. 7).

Discussion

The involvement of CTS *and beta-oxidation in controlling germination potential*

The importance of *CTS* in the regulation of both germination potential and seedling establishment has been

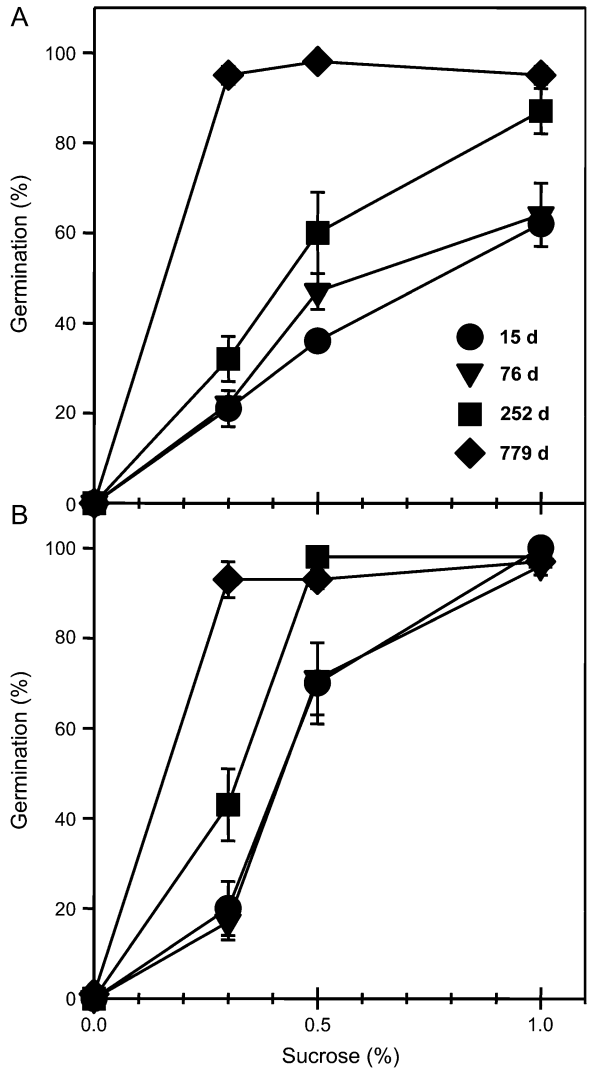

Fig. 6. Effect of sucrose and nitrate on the germination potential of *ttg1-1 cts-1* double mutant seeds assayed at increasing times of dry storage. (A) Germination potential (%) on water agarose media. (B) Germination potential (%) on water agarose media supplemented with 25 mM KNO_3. In each case, seed samples dry stored for increasing time periods (indicated in the figure in days, d) were analysed.

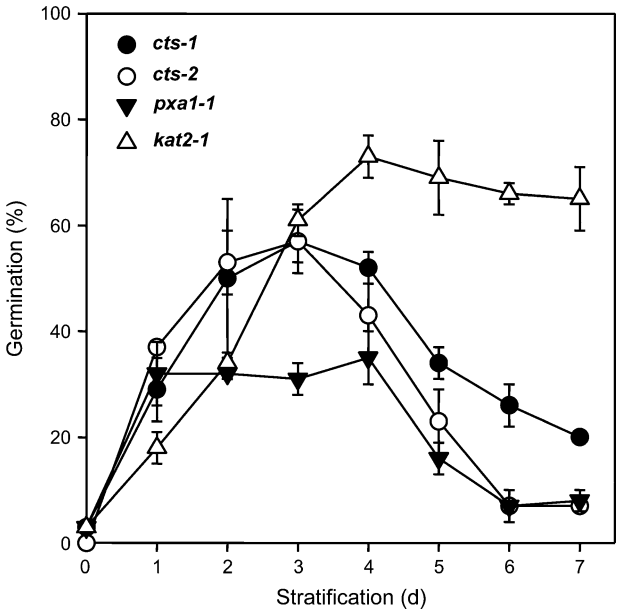

Fig. 7. Effect of stratification on germination potential of *cts* alleles and *kat2-1*. Seeds were incubated in the dark at 4 °C for the indicated periods of time and then transferred to standard germination conditions. Germination was scored 7 d after transfer.

described previously by several groups of workers (Hayashi *et al.*, 1998, 2002; Zolman *et al.*, 2001; Footitt *et al.*, 2002). Work analysing the function of other peroxisome-related enzymes (in particular those encoded by *KAT2*, *LACS6/LACS7*, *ACX1/ACX2*, and *CSY2/CSY3*) has also demonstrated the importance of peroxisomal beta-oxidation during seedling establishment in *Arabidopsis* (Hayashi *et al.*, 1998; Germain *et al.*, 2001; Fulda *et al.*, 2004; Pinfield-Wells *et al.*, 2005; Pracharoenwattana *et al.*, 2005). Differences in assay conditions in different laboratories have led to different phenotypes being reported for mutant alleles of these genes. Germination potential is highly influenced by genotype×environment (G×E) interactions, and analysis of germination *per se* is confounded by after ripening time and dormancy capacity of stored seeds, again highly influenced by G×E interactions (Bewley and Black, 1994; Farnsworth, 2000; Donohue *et al.*, 2005). Therefore, in this paper, a physiological approach has been used to compare directly the dormancy capacity/germination potential of available alleles of important loci related to peroxisomal beta-oxidation in order to understand better the relative contributions of each encoded protein to the control of germination. Defined germination/plant growth/seed storage conditions were utilized to analyse germination potential of seed lots. Previous work has shown that germination potential (i.e. the relationship between dormancy status and after-ripening) appears to be activated by an increase in sensitivity to germination-enhancing stimuli (Hilhorst and Karssen, 1992). The three *cts* mutant alleles tested all showed a marked reduction in germination potential, regardless of germination media, confirming the importance of this gene in controlling germination. The germination potential of *kat2-1* seeds was reduced in comparison to WT, although it was greater than that of *cts* alleles. Similar results have recently been reported for *cts-2*, *kat2-1*, and the *acx1-1 acx2-1* double mutant (Pinfield-Wells *et al.*, 2005). For *cts* alleles and *kat2-1*, establishment of the low percentage of germinated seeds only occurred in the presence of exogenous sucrose, which provides an energy source for seedling establishment in the absence of endogenous substrates accessible for energy production. The small percentage of germination seen for these mutant seeds may be related to genetic redundancy for KAT2, due to very low level expression of KAT5 (Germain *et al.*, 2001), or to biochemical 'leakage' of CTS transport function (there is only one *CTS*

gene in *Arabidopsis*; Footitt *et al.*, 2002), via passive transport across the peroxisomal membrane (as discussed in Theodoulou *et al*., 2005). In future, characterization of CTS and KAT2/5 substrate specificity and analysis of novel mutant alleles providing more subtle changes to protein structure/function will help to elucidate further their respective functions associated with germination.

Beta-oxidation may fulfil one (or more) of three possible functions in germination: provision of energy by lipid catabolism, synthesis of a factor which promotes germination or, alternatively, removal of a factor which inhibits germination (Footitt *et al.*, 2002; Pinfield-Wells *et al.*, 2005; Pracharoenwattana *et al.*, 2005). Mutants that show a similar (very low germination potential even in the presence of sucrose) phenotype to *cts* alleles, are *kat2-1* (this study and Pinfield-Wells *et al*., 2005), an *acx1-1 acx2-1* double mutant (Pinfield-Wells *et al.*, 2005), and a *csy2-1 csy3-1* double mutant which lacks the seedling-expressed glyoxysomal citrate synthase (Pracharoenwattana *et al.*, 2005). All these mutants are defective in beta-oxidation as judged by the failure to break down stored TAG, the retention of lipid bodies, and the accumulation of long-chain acyl CoAs. Strikingly, the *lacs6-1 lacs7-1* double mutant was completely unaffected in germination potential in comparison to WT (although the speed of germination was significantly lower for *lacs6-1 lacs7-1* seeds in comparison to WT), while seedlings were only able to establish in the presence of sucrose, as previously shown (Fulda *et al.*, 2004). As the *lacs6-1 lacs7-1* double mutant also fails to break down TAG and accumulates acyl CoAs, it appears that neither TAG breakdown nor acyl CoA accumulation are important for germination potential. Since the provision of exogenous sucrose cannot significantly increase the germination potential of after-ripened *cts1*, *cts2*, *pxa1*, or *kat2* the hypothesis that energy derived from lipid mobilization is important for germination is unlikely, although clearly this is an essential function for seedling establishment once seed germination has taken place. Analysis of sucrose levels in *cts/kat2-1/lacs6-1lacs7-1* seeds following imbibition indicated a similar slow breakdown that was different from that of WT (which showed a transient sucrose peak related to breakdown of stored lipid). This suggests that endogenous sucrose is not an essential component for germination, since only *lacs6-1 lacs7-1* seeds germinated. The hypothesis that either a germination-promoting molecule is produced or an inhibitor is removed via beta-oxidation appears more likely (Footitt *et al.*, 2002; Pinfield-Wells *et al.*, 2005; Pracharoenwattana *et al.*, 2005). From the data available it would appear that such a molecule would be transported into peroxisomes by CTS, acted upon by ACX1 or ACX2 and thiolase and that the function of peroxisomal CSY, presumably for the removal of acetyl CoA generated by thiolase, is also required. However, activation of the hypothetical molecule by LACS6 and/or 7 is not required. One candidate is JA, the precursors of which, OPDA and OPC8:0, are activated by distinct peroxisomal LACS (Schneider *et al.*, 2005). Recently it was shown that CTS is involved in the accumulation of JA, probably by mediating peroxisomal import of the precursor OPDA, which is then converted to JA by beta-oxidation (Theodoulou *et al.*, 2005). The JA biosynthesis mutant *opr3-1* (encoding a peroxisomal enzyme functioning downstream of CTS) showed no alteration in germination potential or establishment, suggesting that JA is not required for germination unless seeds contain an alternative OPR isoform which can accept the correct stereoisomer of OPDA. However, the characterization of recombinant OPR isoforms suggests that this is unlikely (Schaller *et al.*, 2000). A role for JA is also precluded by the observation that the single *acx1* mutant, which is compromised in JA production, does not have a reported germination phenotype (Pinfield-Wells *et al.*, 2005). Alternatively, beta-oxidation may be required to remove factors that inhibit germination (Baker *et al.*, 2006). Clues to the identity of such molecules may come from the characterization of the substrate specificity and mutant phenotypes of other members of the AAE (acyl enzyme activating) family (Shockey *et al.*, 2003) which include as yet uncharacterized members with putative peroxisome targeting signals (Schneider *et al.*, 2005).

The relationship between CTS function and after-ripening

Reduced germination potential may be due to either increased dormancy (i.e. the requirement for after-ripening) or lack of germination competence following after-ripening through a block in the germination process itself. In either case, seeds remain in germination phase two. Previously it had been shown that *cts-1* mutant seeds demonstrate some characteristics of dormancy (including similar protein profiles; Russell *et al.*, 2000). To define the germination-associated function of CTS, it is important to know whether this gene controls after-ripening status or downstream post-imbibition pathways within the germination phase, about which little is known (Bewley, 1997). The *ttg1-1 cts-1* double mutant was demonstrated to show after-ripening, since stored seeds showed progressively higher germination potential, which was enhanced by both sucrose and nitrate. This indicated that the block to germination in *cts* alleles is post-after-ripening, confirming the role of CTS in germination-associated pathways. It is still unclear why sucrose can completely rescue germination of after-ripened *ttg1-1 cts-1* mutant seeds if beta-oxidation associated with germination is not simply substituting for an energy requirement. However, there has been shown to be a strong interaction between sucrose and ABA during seedling establishment, and for sucrose to suppress germination inhibition by ABA, and so it may be possible that this function also occurs prior to germination (Finkelstein and Lynch, 2000; Finkelstein and Gibson, 2002; Chen *et al.*, 2006).

The partial rescue of germination potential as a transient response of *cts* alleles and *kat2-1* seeds to stratification indicates that stratification, like nitrate, generates a dormancy breaking signal that in the presence of sucrose is able to bypass the *cts*/*kat2-1* lesions. The difference in response of *cts* and *kat2-1* seeds indicates different physiological responses as a result of these mutations, suggesting that, although both loci function in the pathway of beta-oxidation, other functional differences may exist. The transient nature of the stratification response in seeds of *cts* alleles indicates that a germination-related signal is not persistent. This is consistent with there being a time lag between the application and perception of a dormancy-breaking stimulus and ultimate transduction into a germination response (Footitt and Cohn, 1992; Footitt *et al.*, 1995), and that non-saturating dormancy-breaking stimuli generate a physiological but not a germination response (Dedonder *et al.*, 1992). This result also suggests that mechanisms that break dormancy may do so by by-passing the requirement for CTS/KAT2 function in germination initiation.

The *cts* alleles analysed showed intermediate viability and desiccation tolerance when compared with the highly dormant seeds of ecotype CVI. As both physiological characteristics are well known to be associated with dormant seeds, this indicates that although mutant seeds are disrupted in germination-associated progression, aspects of dormant seed physiology are retained, which may reflect the exact stage of germination at which mutant seeds are stalled. Comparisons of the retention of desiccation tolerance showed *cts* alleles to be intermediate between their respective WTs and dormant CVI. In the ecotypes L*er* Col-0 and Ws2, desiccation tolerance was lost coincident with radicle emergence. The decline in desiccation tolerance and subsequent loss of viability seen in the *cts* alleles occurred in the absence of radicle emergence. Use of the dormant CVI and non-dormant WTs of the *cts* alleles provided 'boundary markers' for the retention of desiccation tolerance and viability. This strategy did not reveal a point within the germination phase where *cts* alleles were stalled. However, it did reveal that they are blocked in the germination phase beyond that of dormant seeds, as indicated by the slow loss of both viability and desiccation tolerance. A transcriptome-based analysis of gene expression in mutant seeds in comparison to WT dormant and after-ripened seeds may help to define the developmental status of *cts* seed further.

In conclusion, results have been presented utilizing a comparative physiological genetics approach that provides evidence for the importance of peroxisomal beta-oxidation in the regulation of germination potential, allowing the progression from germination phase two to phase three (radicle emergence). The CTS protein is an essential component in the regulation of this transition, and also influences gene expression profiles associated with the dormancy to germination transition.

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