Growth of Transplastomic Cells Expressing D-Amino Acid Oxidase in Chloroplasts Is Tolerant to D-Alanine and Inhibited by D-Valine[1][W][OA]

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Dual-conditional positive/negative selection markers are versatile genetic tools for manipulating genomes. Plastid genomes are relatively small and conserved DNA molecules that can be manipulated precisely by homologous recombination. High-yield expression of recombinant products and maternal inheritance of plastid-encoded traits make plastids attractive sites for modification. Here, we describe the cloning and expression of a *dao* gene encoding D-amino acid oxidase from *Schizosaccharomyces pombe* in tobacco (*Nicotiana tabacum*) plastids. The results provide genetic evidence for the uptake of D-amino acids into plastids, which contain a target that is inhibited by D-alanine. Importantly, this nonantibiotic-based selection system allows the use of cheap and widely available D-amino acids, which are relatively nontoxic to animals and microbes, to either select against (D-valine) or for (D-alanine) cells containing transgenic plastids. Positive/negative selection with D-amino acids was effective in vitro and against transplastomic seedlings grown in soil. The dual functionality of *dao* is highly suited to the polyploid plastid compartment, where it can be used to provide tolerance against potential D-alanine-based herbicides, control the timing of recombination events such as marker excision, influence the segregation of transgenic plastid genomes, identify loci affecting *dao* function in mutant screens, and develop D-valine-based methods to manage the spread of transgenic plastids tagged with *dao*.

Selectable marker genes provide powerful genetic tools for manipulating plant genomes (Miki and McHugh, 2004; Day and Goldschmidt-Clermont, 2011). High and stable accumulation of high-value products in transgenic plastids (Daniell et al., 2009; Maliga and Bock, 2011), combined with restricted dissemination of plastid transgenes in pollen (Daniell et al., 1998; Ruf et al., 2007; Svab and Maliga, 2007), make plastid genomes an attractive target for modification. Reverse genetics allows site-directed changes to be introduced into the important set of genes present in plastids to study and ultimately improve their function (Whitney et al., 2011; Day, 2012). Positive conditional selectable marker genes allow transgenic cells to divide in the presence of chemicals such as antibiotics or herbicides that inhibit wild-type untransformed cells and underpin the development of herbicide-resistant transplastomic crops (Daniell et al., 1998, 2009; Iamtham and Day, 2000; Lutz et al., 2001; Ye et al., 2001; Dufourmantel et al., 2007; Shimizu et al., 2008). Negative conditional selectable marker genes inhibit the proliferation of transgenic cells when exposed to compounds that have a limited impact on the viability of wild-type cells (Miki and McHugh, 2004). Most commonly, the enzyme product of a negative selection gene converts an exogenous chemical substrate into a toxic product. Negative selectable markers allow genetic restriction technologies to manage the spread of transgenic crops (Daniell, 2002; Hills et al., 2007) and are valuable components of genome manipulation technologies involving recombination (Hardy et al., 2010). Negative selection can be used to promote genome changes by, for example, linking recombination events to the excision of a negative selection marker gene. Dual positive/negative selectable marker genes are particularly versatile tools for genome engineering. An example is the uracil3 (*URA3*) gene marker in *Saccharomyces cerevisiae*, where selection for or against *URA3* encoding orotidine 5′-phosphate decarboxylase has been used to influence the timing of recombination events to manipulate chromosomes and introduce site-directed mutations into *S. cerevisiae* genes (Boeke et al., 1987).

Homologous recombination is an effective tool for genome engineering in bacteria (Link et al., 1997) and *S. cerevisiae* (Boeke et al., 1987) and is the predominant recombination pathway operating in plastids (Day and Madesis, 2007). Plastid genomes are relatively small, sequenced in over 200 species (Jansen and Ruhlman, 2012), polyploid, and are highly suitable targets for genome engineering (O'Neill et al., 2012). Establishing

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a dual-marker system in plastids would allow control over the timing and efficiency of homologous recombination events, such as marker excision between direct repeats (Iamtham and Day, 2000). New positive selection markers not involving antibiotics are candidates for developing herbicide-tolerant transplastomic crops, contained by maternal inheritance of plastids (Daniell et al., 1998). Genetic containment methods require the insertion of a conditional negative selectable marker into the plastid genome and may be desirable in transplastomic "pharma" crops and cells expressing products for medicine (Daniell et al., 2009; Oey et al., 2009a, 2009b; Ruhlman et al., 2010; Gisby et al., 2011). Negative markers inserted into the nucleus would be ineffective for preventing the spread of transgenic plastids because nuclear and chloroplast genes are inherited independently and by different mechanisms.

Nuclear expression of the *Rhodotorula gracilis dao* gene encoding D-amino acid oxidase (DAAO; EC 1.4.3.3) allows positive or negative selection of transgenic plant cells (Erikson et al., 2004). Transgenic nuclear *dao* plants were tolerant to D-Ala and D-Ser but sensitive to D-Val and D-Ile. A reversal of the tolerance pattern was observed in wild-type plants, which were sensitive to D-Ala and D-Ser but tolerant to D-Val and D-Ile. The toxic ketoacids 3-methyl-2-oxobutanoate and 3-methyl-2-oxopentanoate are produced by DAAO-catalyzed deamination of D-Val and D-Ile, respectively (Erikson et al., 2004). Originally developed in Arabidopsis (*Arabidopsis thaliana*), the system has been applied to crops (Lai et al., 2007). Importantly, D-amino acids are relatively cheap and nontoxic (Gullino et al., 1956; Friedman, 1999), thereby facilitating their use outside the laboratory. Cost and safety are important factors for applications as positive selection herbicides to control weeds in transgenic crops and negative selection agents to control the spread of transgenic crops. A dual selectable marker gene has not previously been described in plastids. The bacterial *codA* gene, encoding cytosine deaminase (Mullen et al., 1992), is the sole negative selection marker shown to work in plastids (Serino and Maliga, 1997). Transplastomic cells expressing *codA* are sensitive to the antibiotic 5-fluorocytosine (Serino and Maliga, 1997), which is used to treat fungal infections in humans, with possible side effects including hepatotoxicity (Steer et al., 1972). The relatively high cost of 5-fluorocytosine and its potential toxicity on nontarget organisms would hinder its use as a negative selection agent to manage the spread of transgenic crops expressing *codA*.

Plastids are important centers for amino acid synthesis and metabolism (Lancien et al., 2006), but the impact of D-amino acids on plastids had not been reported previously. Here, we have developed a plastid marker based on the *Schizosaccharomyces pombe dao* gene (Wood et al., 2002) that confers either positive (D-Ala) or negative (D-Val) selection on transplastomic cells. Our results are consistent with D-amino acids being transported into plastids, where D-Ala inhibits one or more plastid functions and D-Val is converted into a toxic product by DAAO. The plastid *dao* gene provides a new versatile marker for plastid genetics, with applications for developing D-Ala-tolerant transplastomic plants, D-Val-based containment of transgenic plastids, and controlling the timing of recombination events in plastid genomes.

RESULTS

Isolation of a *dao* Coding Sequence for Plastid Expression

A *dao* gene was identified in the nuclear genome of the fission yeast *S. pombe* (Wood et al., 2002). The *S. pombe dao* gene has a GC content of 45%, which is closer to the approximately 30% GC content of plastid genes (Shimada and Sugiura, 1991) than the 63% GC content of the *R. gracilis dao* gene (Pollegioni et al., 1997). The 348-amino acid *S. pombe* DAAO protein contains the 24 conserved amino acids critical for DAAO activity (Tishkov and Khoronenkova, 2005). The coding sequence was isolated by PCR and cloned into the *Escherichia coli* pET30 expression vector. The DAAO activity of the *S. pombe* protein was demonstrated by overexpressing the *S. pombe* DAAO gene (*dao*) in *E. coli* and assaying cell extracts. Total soluble protein from isopropyl-L-D-1-thiogalactopyranoside (IPTG)-induced cultures fractionated by SDS-PAGE gave rise to a prominent 35-kD band from induced pET30-*dao E. coli* cultures (Fig. 1). The prominent 35-kD band was not visible in lanes corresponding to pET30 cultures (vector without *dao* gene) or uninduced pET30-*dao* cultures (Fig. 1). The predicted size of the 348-amino acid *S. pombe* DAAO is 39 kD, indicating a higher than expected electrophoretic mobility. Cell extracts containing the overexpressed *S. pombe* DAAO protein showed over 400-fold higher D-amino acid oxidase activities, measured by conversion of D-Ala to pyruvic acid (Oguri et al., 2007), than control extracts

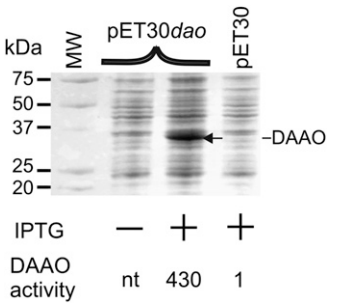

Figure 1. Expression of the *S. pombe dao* gene in *E. coli*. Coomassie blue-stained total soluble protein was from *E. coli* strains fractionated by SDS-PAGE (12% [w/v] polyacrylamide). Lanes corresponding to recombinant pET30-*dao* and empty pET30 vector strains induced (+) or not induced (−) with IPTG are indicated. Relative specific DAAO activities of total soluble protein from *E. coli* strains containing (pET30-*dao*) or lacking (pET30) the overexpressed DAAO protein are indicated. MW, Molecular weight marker; nt, not tested.

lacking the overexpressed *S. pombe* DAAO protein (Fig. 1).

Assembly of the Plastid Transformation Vector

The *S. pombe dao* gene was cloned into a plastid expression cassette containing a plastid *rrn* promoter and *rbcL* ribosome-binding site from tobacco (*Nicotiana tabacum*) and terminated by a 3′ regulatory element from the *Chlamydomonas reinhardtii rbcL* gene (Supplemental Fig. S1). The *dao* expression cassette was combined with the plastid *aadA* marker gene and inserted between the *rbcL* and *accD* genes in a previously cloned 7.2-kb sequence of tobacco plastid DNA (Sugiura et al., 1986). The resulting 13.9-kb pUM20-*dao* plastid transformation vector (Fig. 2) contains 5.7- and 1.5-kb arms of tobacco plastid DNA for targeted integration into the plastid genome.

Molecular Analyses of Plastid Transformants

Total leaf DNA from four plastid transformants (pt^{dao} lines 1–4) isolated from independent transformation events was analyzed by DNA-blot analysis. *Hin*dIII sites located in wild-type plastid DNA (Fig. 3A) lie outside the targeting arms present in plastid transformation vector pUM20-*dao* (Fig. 2). The *rbcL* gene is located on an 11.5-kb *Hin*dIII fragment in wild-type plastid DNA (Fig. 3A) and a 7.0-kb fragment in transgenic plastid DNA (Fig. 3B). An *rbcL* hybridization probe showed replacement of the 11.5-kb wild-type band (Fig. 3C, lane 1) by the predicted 7.0-kb

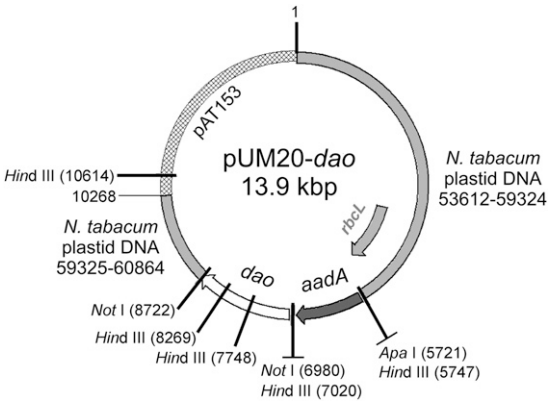

Figure 2. Map of tobacco plastid transformation vector pUM20-*dao* (13.9 kb). The vector targets the integration of *aadA* and *dao* genes to the *Aoc*I site located at base 59,324 of the 155,943-bp tobacco plastid genome (Yukawa et al., 2005). The *Aoc*I site is located in the intergenic region between the *rbcL* and *accD* (data not shown) genes. Shown are tobacco plastid-targeting arms, *aadA*, *dao*, native *rbcL* genes, and the vector pAT153 (Twigg and Sherratt, 1980). The *aadA* and *dao* expression cassettes include the *rrn* promoters, *rbcL* ribosome-binding sites, and 3′ regulatory regions. The *Hin*dIII site at 8,269 bp is located outside the *dao* coding region before the 3′ untranslated region from the *C. reinhardtii rbcL* gene.

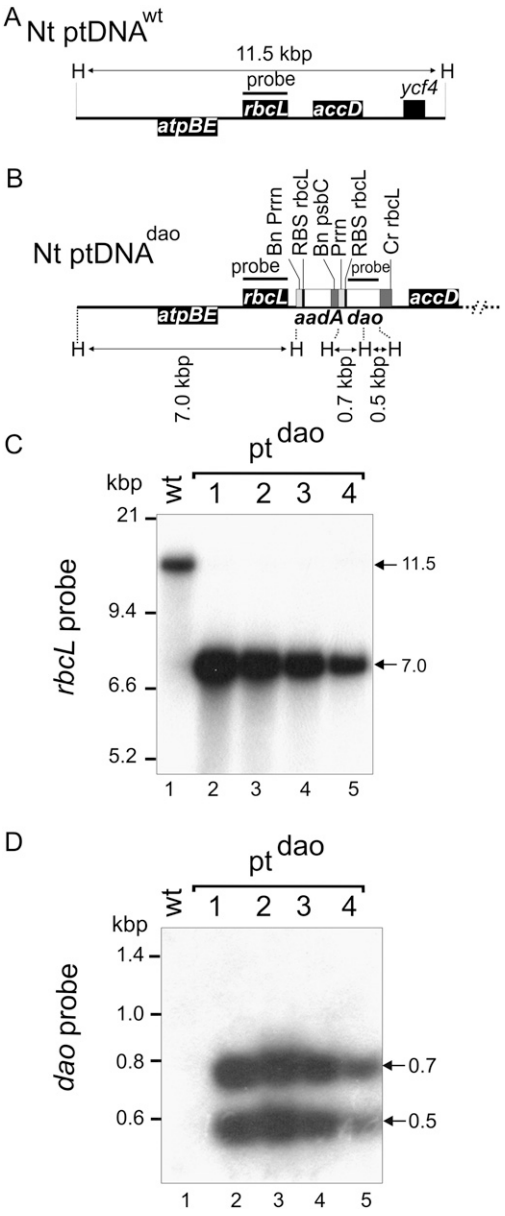

Figure 3. Targeted integration and homoplasmy of the plastid *dao* marker gene. A and B, Maps of tobacco wild-type DNA (Nt $ptDNA^{wt}$; A) and transgenic plastid DNA (Nt $ptDNA^{dao}$; B) showing native plastid genes (dark boxes) and transgenes (light boxes) at the integration site. Promoter (*rrn*), ribosome-binding site (RBS), and *psbC* and *rbcL* 3′ regulatory sequences for *aadA* and *dao* are shown. Tobacco regulatory elements were used unless indicated as follows: *B. napus* (Bn) or *C. reinhardtii* (Cr). *Hin*dIII (H) sites and hybridization probes are located. C and D, DNA-blot analyses of *Hin*dIII digests with the indicated probes. Shown are wild-type and independent transplastomic (pt^{dao}) lines, molecular weight (MW) standards, and band sizes.

band in transgenic lanes (Fig. 3C, lanes 2–5). This confirms targeted integration and homoplasmy of transgenic plastid genomes in the four pt^{dao} transplastomic lines studied. A probe containing only *dao* coding sequences hybridized to the predicted 0.5- and 0.7-kb *Hin*dIII bands in digests of transformant DNA

(Fig. 3D, lanes 2–5), which were not present in the wild-type lane (Fig. 3D, lane 1).

Expression of the *dao* gene in two independent lines (lines 1 and 2) was studied by RNA-blot analysis. Blots containing leaf RNA from wild-type and transplastomic leaves fractionated on denaturing formaldehyde gels (Fig. 4A) were hybridized to *rbcL* and *dao* probes. The *rbcL* probe hybridized to a single 1.8-kb RNA band in the wild-type lane (Fig. 4B, lane 1), corresponding to the monocistronic *rbcL* transcript. The 1.8-kb *rbcL* band is present in transplastomic *dao* lanes (Fig. 4B, lanes 2–4), which also contain larger 2.9- and 4.5-kb RNA bands resulting from read-through transcription into the downstream foreign genes (Fig. 4D). The *dao* probe hybridizes to a 1.5-kb monocistronic band and to larger 2.7- and 4.5-kb RNA bands in transplastomic lanes (Fig. 4C, lanes 2–4). These large transcripts are likely to be initiated at the upstream promoters driving the expression of *rbcL* and *aadA* (Fig. 4D).

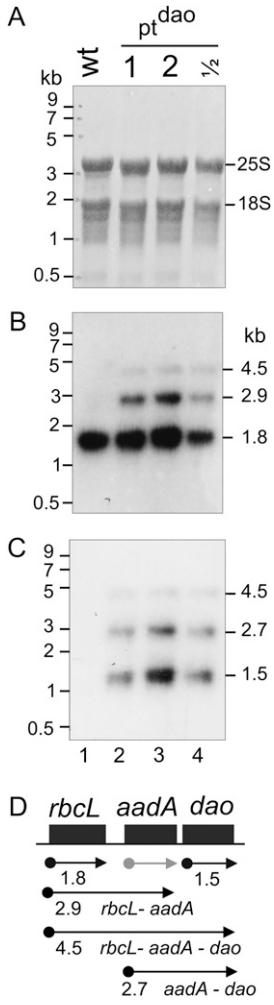

Figure 4. Transcription of *dao* in transplastomic plants. Leaf RNA from the wild type (wt) and two pt^{dao} transplastomic lines fractionated by denaturing electrophoresis was transferred to nylon membranes. A, RNA stained with methylene blue. B, *rbcL* probe hybridization. C, *dao* probe hybridization. D, Map locating transcripts detected by probes. RNA molecular weight standards and sizes of RNA bands detected on blots are shown.

Response of Transplastomic pt^{dao} Cells and Plants to D-Amino Acids

Transplastomic pt^{dao} plants resembled wild-type plants in appearance and were fertile, giving rise to 100% maternal transmission of resistance conferred by transgenes. The next seed generation (T1) was tested against D-amino acids. The intracellular target(s) of D-Ala is not clear, with one possible target relating to inhibition of tRNA function (Erikson et al., 2004), presumably in one or more of the cytosolic, plastidic, or mitochondrial subcellular compartments carrying out protein synthesis. The response of wild-type and pt^{dao} transplastomic cells to D-amino acids was determined by their impact on the growth of cells and plants in vitro and in soil. Three assays were used: (1) division of cells and formation of shoots from leaf explants in vitro; (2) germination and growth of seedlings in vitro; and (3) growth of seedlings in soil. In the first assay, leaf pieces from the wild type and two pt^{dao} transplastomic lines (lines 1 and 2) were placed on revised medium for organogenesis (RMOP; Svab and Maliga, 1993) containing D-amino acids. Leaf explants on day 1 (Fig. 5A) and 56 d (Fig. 5B) after being placed on media containing D-Ala illustrate the differing responses of wild-type and transplastomic cells. Leaf cells from transplastomic *dao* plants divided and regenerated into green shoots on D-Ala medium, whereas wild-type leaf explants bleached and remained quiescent (Fig. 5B). The reverse was observed on D-Val medium, where cells in wild-type leaf explants proliferated and formed shoots whereas transplastomic *dao* leaf explants bleached and did not divide (Fig. 5C). No restriction of growth was observed for transplastomic or wild-type leaf pieces on control RMOP plates lacking D-amino acids (Fig. 5D).

In the second assay, seeds from wild-type and transplastomic plants were germinated on Murashige and Skoog (MS) medium containing D-amino acids. Following germination on D-Ala plates, wild-type seedlings arrested at the cotyledon stage and turned brown and necrotic (Fig. 5E, left). Transplastomic seedlings formed true leaves on D-Ala medium but exhibited limited root growth and reduced leaf growth (Fig. 5E, right). The *dao* gene was expressed from the plastid *rrn* promoter, which is poorly expressed in roots relative to leaves (Zubko et al., 2004), reducing the resistance of this organ to D-Ala. Reduced root growth and function are probably responsible for the stunted phenotype of transplastomic pt^{dao} seedlings on D-Ala medium. On D-Val medium, wild-type seedlings formed roots and grew well (Fig. 5F, left). In contrast, transplastomic pt^{dao} seedlings were severely inhibited by D-Val (Fig. 5F, right). The fresh weight of wild-type

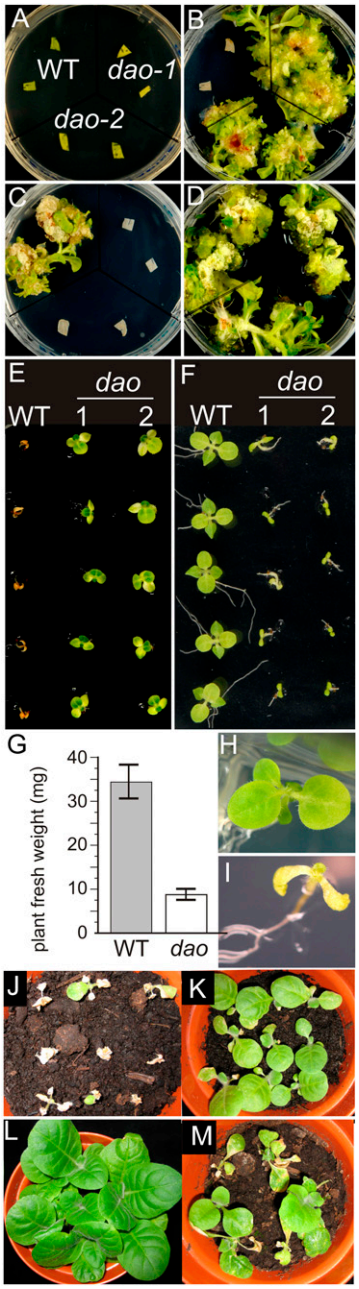

Figure 5. Tolerance of wild-type (WT) and pt^{dao} transplastomic plants to D-amino acids. A to D, Leaf explants were arranged on RMOP regeneration medium as indicated on day 1 (A) and after 56 d on 2.5 mM D-Ala (B), 6 mM D-Val (C), or no added D-amino acids (D). E and F, Seedlings photographed at 20 d post germination on MS medium with 10 mM D-Ala (E) or 5 mM D-Val (F). G, Fresh weight of aerial plant parts 55 d post germination on 2 mM D-Val medium. Values shown are means of eight plants. H and I, Wild-type plant (H) and pt^{dao} plant (I) 55 d post germination on 2 mM D-Val medium. J and K, Wild-type plants (J) and pt^{dao} plants (K) sprayed with 50 mM D-Ala on days 1 and 5 and photographed on day 8. L and M, Wild-type plants (L) and pt^{dao} plants (M) sprayed with 50 mM D-Val on days 1, 5, and 9 and photographed on day 19. Each pot contained nine seedlings with two true leaves on day 1.

plants grown on D-Val medium was much higher than that of pt^{dao} plants (Fig. 5G). At this stage, wild-type seedlings had developed into plants with several leaves (Fig. 5H) whereas pt^{dao} seedlings remained at the cotyledon stage of growth with stunted and distorted first true leaves (Fig. 5I). The pt^{dao} seedlings formed roots on D-Val medium (Fig. 5I), which is consistent with a lack of expression of *dao* in roots. Reduced activity of the plastid *rrn* promoter driving the expression of *dao* in roots would lower D-amino acid oxidase levels and the conversion of D-Val to toxic 3-methyl-2-oxobutanoate.

In the third assay, seedlings growing in soil with cotyledons and the first two true leaves were sprayed with solutions of 50 mM D-Ala or 50 mM D-Val. D-Ala had a severe negative impact on the growth of wild-type seedlings, resulting in shrinkage and browning of leaves at 8 d post spraying (Fig. 5J). In contrast, transplastomic pt^{dao} seedlings were relatively tolerant to D-Ala sprays (Fig. 5K). The opposite was observed for D-Val sprays. After 19 d, the pt^{dao} transplastomic plants were stunted, with most exhibiting leaf damage (Fig. 5M). In contrast, wild-type plants were healthy (Fig. 5L), demonstrating the selective damage imposed by D-Val on transplastomic plants growing in soil.

DISCUSSION

Expression of a *dao* gene from *S. pombe* in tobacco chloroplasts gave rise to transplastomic pt^{dao} plants that resembled wild-type plants in growth and appearance. The phenotype of pt^{dao} plants was conditional on exposure to D-amino acids. Growth of pt^{dao} cells was tolerant to D-Ala but inhibited by D-Val, whereas wild-type cells were inhibited by D-Ala and tolerant to D-Val. The results are consistent with the import of D-amino acids into plastids and indicate that a major target for D-Ala, which is toxic to wild-type plants, is likely to be located in plastids. Deamination of D-Val to 3-methyl-2-oxobutanoate takes place within the plastids of pt^{dao} cells. Compartmentation of 3-methyl-2-oxobutanoate production within plastids does not prevent its inhibitory effect on plant growth. The *S. pombe dao* plastid marker described here is, to our knowledge, the first dual positive/negative selectable marker shown to be operational in plastids. Selection does not involve antibiotics, which is of added benefit for the plastid transformation toolbox (Day and Goldschmidt-Clermont, 2011).

Negative conditional selection against transplastomic plants containing *dao*-tagged plastid genomes with D-Val provides a potential management tool to limit the spread of plastid transgenes. Both D-Ala and D-Val are relatively low-cost D-amino acids. In heteroplasmic plants, negative selection against *dao*-tagged plastid genomes could be used to influence cytoplasmic sorting and the attainment of homoplasmy. For applications in which *dao* is not required in transplastomic plants, it can be used to control marker excision between direct repeats.

Marker excision by spontaneous recombination between direct repeats is an effective tool for removing marker genes from plastid genomes (Iamtham and Day, 2000; Kode et al., 2006). Split herbicide resistance genes enable positive selection using herbicide to restore gene function by recombination and excision of the intervening *aadA* antibiotic selection marker (Dufourmantel et al., 2007). Inclusion of *dao* in an excision cassette containing *aadA* allows negative selection against transgenic genomes containing marker genes and promotes the accumulation of marker-free plastid genomes. In situations where *dao* is the sole marker located between direct repeats, it can be used to either maintain the nonrecombined form by positive selection or promote excision by negative selection. This use of dual selectable marker genes can be used to introduce site-directed mutations into genomes by recombination, without retention of foreign sequences (Boeke et al., 1987; Hardy et al., 2010). This recombination route allows the introduction of single-base changes into a wild-type plastid genome to modify and ultimately improve the properties of plastid genes. Negative selection markers such as *dao* and *codA* (Serino and Maliga, 1997) provide opportunities to develop mutant screens to identify nuclear gene products involved in the expression and maintenance of the marker. Mutation or down-regulation of nuclear genes required for *dao* function would give rise to cells that survive negative selection. This would be more feasible in diploid species tractable to mutant screens and plastid transformation, such as petunia (*Petunia hybrida*; Zubko et al., 2004) and *Nicotiana sylvestris* (Maliga and Svab, 2011).

Positive selection with D-Ala can be used to maintain *dao*-tagged plastid genomes in transplastomic plants. It can also be used to maintain unstable recombination substrates leading to excision of *dao*. The tolerance of *dao* transplastomic plants to foliar sprays of D-Ala provides scope for developing a D-Ala-based herbicide that would selectively inhibit weed growth in transplastomic crops. Compared with the *aadA* marker gene, the *dao* marker is not an efficient primary marker for isolating plastid transformants (data not shown). This is also true of a number of other positive selection markers expressed in plastids, including those conferring resistance to herbicides (Lutz et al., 2001) or actinonin (Fernández-San Millán et al., 2011). This can be explained by the idea that these herbicides kill cells containing a small fraction of transformed plastids (Ye et al., 2003). In contrast, cells with one or a few transgenic plastids are viable in the presence of positive selection agents such as spectinomycin (Svab and Maliga, 1993), allowing the transgenic resistant plastids to divide and increase in number. Tolerance to herbicides is achieved once sufficient transgenic plastids are present in a cell. This phenomenon of cellular lethality to positive selection agents based on the level of heteroplasmy is a unique feature of organelle genetics and has given rise to the concept of primary and secondary markers in plastid transformation (for review, see Day and Goldschmidt-Clermont, 2011). We have found the plastid *dao* marker gene to be a good marker for secondary selection once resistant green cells are identified using *aadA*-based spectinomycin selection (Svab and Maliga, 1993). True transformants remain green and proliferate, while spontaneous spectinomycin-resistant mutants are rapidly bleached and die. Once resistant shoots have been isolated, the *dao* gene can be maintained by D-Ala-based selection and the *aadA* removed by methods involving native plastid pathways (Iamtham and Day, 2000; Ye et al., 2003) or foreign site-specific recombinases (Corneille et al., 2001; Hajdukiewicz et al., 2001; Lutz and Maliga, 2007).

MATERIALS AND METHODS

Vector Construction

Standard cloning methods were used (Sambrook et al., 1989) using One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen). Primers DAO-F (5′-GGGATCCATATGACTAAGGAAAATAAGCCAAGAG-3′) and DAO-R (5′-CCTGCAGTTAAGCCAATTTGATTTTAGGAAG-3′) were used to PCR amplify the DAAO coding sequences using *Schizosaccharomyces pombe* genomic DNA. The 1,047-bp coding sequence cloned in pGEM T-Easy (Promega), flanked by engineered *Bam*HI and *Pst*I sites, was identical to sequence CU329672 (Wood et al., 2002), apart from replacement of the terminal TAG stop codon with TAA. The sequence encodes a predicted D-amino acid oxidase of 348 residues (accession no. CAB40174; GI 4581517). The sequence of *dao* cloned into the plastid expression cassette pUM31 is shown in Supplemental Figure S1 (EMBL accession no. HF545301). The *dao* coding sequence was inserted as an *Nde*I-*Sac*I fragment into pET30b (Invitrogen) and expressed in *E. coli* BL21(DE3)pLysS cells (Invitrogen). The *aadA* marker gene flanked by a *Brassica napus rrn* promoter, tobacco (*Nicotiana tabacum*) *rbcL* ribosome-binding site, and *B. napus psbC* 3′ untranslated region has been described (Goldschmidt-Clermont, 1991; Iamtham and Day, 2000; Zubko et al., 2004). The *aadA* and *dao* genes in plastid expression cassettes were cloned between *Apa*I and *Not*I sites in a polylinker inserted in an *Aoc*I site (Zubko et al., 2004) present in a 7.2-kb tobacco plastid DNA sequence (Sugiura et al., 1986) cloned in pAT153 (Twigg and Sherratt, 1980). The *Aoc*I site is located at position 59,324 of the 155,943-bp tobacco plastid genome (accession no. Z00044; Shinozaki et al., 1986; Yukawa et al., 2005). The map of plastid transformation vector pUM20-*dao* is shown in Figure 2.

Nucleic Acid-Blot Analyses

Methods for DNA- and RNA-blot preparation and hybridization have been described (Madesis et al., 2010). RNA molecular standards were from New England Biolabs. The *dao* gene probe was an agarose gel-purified fragment of the 1,047-bp coding sequence excised from the recombinant pGEM T-Easy vector (see above). The *rbcL* probe was prepared from tobacco plastid plasmid pTB27 (Sugiura et al., 1986) using primers rbcl-F (5′-ATGTCACCACAAACAGAGACTA-3′) and rbcl-R (5′-TTACTTATCCAAAACGTCCACT-3′), and the resulting 1,434-bp PCR product was gel purified. Following probe hybridization, DNA blots were washed in 0.1× SSC (1× SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) and 0.1% (w/v) SDS at 60°C. RNA blots were washed in the same solution at 50°C.

Expression of Recombinant DAAO in *E. coli*

E. coli BL21(DE3)pLysS strains containing pET30-*dao* and pET30b (empty vector control) were grown at 37°C in Luria-Bertani medium supplemented with 40 mg L^{-1} kanamycin and 30 mg L^{-1} chloramphenicol (Sambrook et al., 1989). IPTG (1 mM) induction was for 2 h once bacterial cells reached an optical density at 600 nm of 0.4 (BioPhotometer Spectrophotometer; Eppendorf). Sedimented pET30-*dao* induced and uninduced cells were concentrated 20-fold, relative to the original culture volume, in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% [v/v] glycerol, 3% [w/v] SDS, 5% [v/v] β-mercaptoethanol, and 0.1% [w/v] bromophenol blue), placed in a boiling-water bath (5 min),

and 5 μL was fractionated by SDS-PAGE on 12% (w/v) polyacrylamide gels (4% [w/v] stacking gel) alongside prestained Precision plus molecular weight marker (Bio-Rad) and stained with Coomassie Brilliant Blue as described (Madesis et al., 2010). For DAAO enzyme assays, induced pET30b-*dao* and control (pET30b empty vector) *E. coli* strains were concentrated 10-fold in cell extract buffer (0.1 M Tris, pH 8, 1 mM EDTA, 0.15 M KCl, 5% glycerol, and 0.6% Triton X-100), lysed by sonication, and sedimented (5,000 rpm; Sorvall SS34 rotor), and the supernatant was used for assays. DAAO enzyme activities were determined according to Oguri et al. (2007) at 37°C including D-Ala (Sigma-Aldrich) and 2,4-dinitrophenylhydrazine (Sigma-Aldrich) in the assays and measuring absorbance of the 2,4-dinitrophenylhydrazine-pyruvic acid product at 445 nm. The protein content of the extracts was estimated using the Bio-Rad DC protein assay to determine specific activity.

Isolation and Analysis of Transplastomic Plants

Homoplasmic plastid transformants were isolated in tobacco 'Wisconsin 38' using spectinomycin and streptomycin selection (each at 0.5 g L^{-1}) and two rounds of selection following particle bombardment (Iamtham and Day, 2000). For growth assays, surface-sterilized seeds were germinated on MS medium (Murashige and Skoog, 1962). The aerial parts of young plants were used for determining fresh weight. Leaves from aseptic plants propagated in vitro were placed on RMOP regeneration medium (Svab and Maliga, 1993) with or without D-amino acids for shoot regeneration assays (12-h days, 80–120 μE m^{-2} s^{-1} at 25°C). D-Amino acids were purchased from Sigma-Aldrich. D-Amino acid solutions (50 mM) containing Tween (0.1%, v/v) were used to spray 10-cm pots containing nine seedlings with cotyledons and two true leaves growing in soil at a dose of 2.5 mL per 10-cm pot (12-h days, 150 μE m^{-2} s^{-1} at 24°C).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number HF545301.

Supplemental Data

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

Supplemental Figure S1. Sequence of plastid expression cassette pUM31-*dao*.

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