SELECTIVE PLANT GROWTH USING D-AMINO ACIDS

By

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ABSTRACT OF THE THESIS

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Selectable marker genes are essential for the efficient selection of transgenic plants. Heterologous genes used as markers typically encode enzymes that neutralize a toxic compound, allowing for positive selection of plants containing the marker. We report on the efficacy of a novel marker gene system that exploits the varying phytotoxicity of D-amino acids and their oxidative deamination products. Our investigation of a putative *DAAO* gene from *Schizosaccharomyces pombe* reveals sufficient oxidative deaminase activity to confer D-alanine and D-serine tolerance to transgenic *Arabidopsis thaliana* plants carrying the heterologous sp*DAAO* gene. We have demonstrated that the sp*DAAO*, when used in conjunction with D-alanine, allows for the positive selection of primary transformants at levels comparable to hygromycin. Additionally, our selection scheme carries the potential for negative selection in the presence of different selection substrates, such as D-valine or D-isoleucine. This attribute, known as conditional selection would provide particular utility to applications involving site-specific recombinase mediated marker gene removal.

Table of Contents

List of Figures and Tables

Introduction

Selectable Marker Genes In Plant Transformation. Selectable markers in plant transformation confer antibiotic or herbicide resistance to transformed cells, which develop into readily identifiable transgenic plants. Selection pressure is generally applied in regeneration media. The antibiotics and herbicides used in conjunction with marker genes typically impart negative selection, resulting in the abnormal or arrested development of untransformed plants. As part of any plant transformation cassette, selectable marker genes are integrated, along with the transgene of interest, into the native DNA of the plant cell. (1)

Marker genes used in positive selection express heterologous proteins that inactivate or degrade the antibiotic or herbicidal selective agent. A limited number of bacterial genes are well established as effective selectable markers for the positive selection of transgenic plants. A 2002 evaluation of several peer-reviewed plant journals found that over 90% of the studies employed selection on kanamycin, hygromycin, or phosphinothricin. (1)

Kanamycin and hygromycin B are antibiotics that target prokaryotic mechanisms, giving them a dual role in medicine and biotechnology. (2) They are both therapeutic antibiotics used to combat bacterial infection, and their effect on the translation machinery of chloroplasts and mitochondria make them valuable selection agents against plants.

Kanamycin was first isolated from the soil bacterium *Streptomyces kanamyceticus.* It is one of several aminoglycoside compounds which block prokaryotic protein synthesis by binding the 30S ribosomal subunit. Aminoglycoside activity is

deactivated via phosphorylation by neomycin phosphotransferase II (NPTII). NPTII activity is expressed in plants from the *nptII* gene native to *Escherichia coli.* (3) In addition to kanamycin, other commonly used aminoglycoside antibiotics include gentamycin, neomycin, and streptomycin.

Hygromycin B is an antibiotic of the aminocyclitol group, and severely inhibits cell expansion in developing plants, resulting in the reduced hypocotyl of wild-type seedlings relative to unselected controls. (4) Hygromycin phosphotransferase activity from the *E.coli* gene *aphIV* restores hypocotyl growth, enabling selection in plant transformation. (5) Hygromycin B has been shown to induce apoptosis in human cancer cells, and has some limited clinical use as a chemotherapeutic agent (6).

Phosphinothricin (PPT) is the ammonium salt of glufosinate, a glutamine analog. PPT has a broad-spectrum herbicidal effect, targeting nitrogen assimilation in plants through its inhibition of glutamine synthase. Acetylation of PPT converts it into a non-toxic form. PPT acetyltransferase activity is conferred to the plant from the *bar* gene of *Streptomyces hygroscopicus.* (7)

Negative selection schemes allow for the positive selection of cells lacking a particular gene. They are facilitated by the expression of counter-selectable marker genes, which results in cell death or abnormal plant morphology. The cytosine deaminase gene *codA* from *E.coli* provides negative selection when used in conjunction with 5-fluorocytosine (5-FC), a non-toxic substrate. The functional expression of *codA* from the plant converts 5-FC into 5-fluorouracil, whose toxicity is sufficiently lethal to provide counter-selection. (8) Efficient negative selection using the *codA* counter-selectable gene has been demonstrated in monocots and dicots (9).

Issues With Plant Selectable Markers. The most commonly used selectable marker systems have proven to be reliable and efficient indicators of transformation events. However, there are theoretical problems related to the safety and environmental impact of the selective agents used, and concerns associated with marker genes remaining in the transgenic plant. (1)

The propagation of resistant bacterial strains is an inherent risk coupled with any antibiotic usage. The antibiotics commonly used in a laboratory setting to identify transformation events also play therapeutic roles in combating human bacterial infection. (10) Hygromycin B requires particular caution owing to its demonstrated apoptotic effects on mammalian cells. (11)

The presence of selectable marker genes in the ecosystem carry some risk of the antibiotic or herbicide resistance trait spreading beyond the host. Transgenic pollen may escape to other fields, contaminating their wild-type counterparts. Interspecific hybridization may result in the expression of broad-spectrum herbicide resistance in weed species. (12) Antibiotic resistance could also be transferred to microbial or mammalian cells by means of horizontal gene transfer. In a study using transgenic tobacco, the relocation to bacteria of an antibiotic resistance gene was accomplished, albeit with homologous recombination facilitated. (13) In another study, plasmid DNA fed to mice turned out to be detectable within their cells. (14) Therefore, the possibility of genetic material moving from the plant into soil-borne pathogens or intestinal microorganisms cannot be excluded. Moreover, the protein products expressed from selectable marker genes may in themselves cause an allergic reaction to the consumer. (15)

D-Amino Acids. With the exception of glycine, each amino acid exists in one of two stereoisomeric forms based on the position of side groups relative to the chiral carbon. The two forms have different optical properties, and are accordingly designated levorotatory (L) or dextrorotatory (D).

L-amino acids play the principal role in metabolism and protein composition of all organisms. The biochemical pathways responsible for the synthesis of L-amino acids involve the transfer of an amino group onto a 2-oxocarboxylic intermediate. This reaction, catalyzed by aminotransferases, exists in all kingdoms and is highly stereospecific. (16)

D-amino acids have a much more limited occurrence in nature than their L stereoisomers. D-amino acids appear to have some structural and signaling functions in both prokaryotes and eukaryotes. D-alanine and D-glutamate are major components of the peptidoglycan layer in bacterial cell walls. (17) D-serine is present at high levels in the human brain, where it functions as a neurotransmitter. (18) Metabolic activity involving D-amino acids has not been observed in the plant kingdom. (19) However, their presence has been detected in higher plants like peas, barley, hops, and tobacco, where they exist in amounts 0.5% to 3% relative to L-amino acids. (20)

The presence of D-amino acids in plants is attributable to racemase activity or the uptake of microbial D-amino acids from the soil. (21) Amino acid racemases catalyze the inversion of L-amino acids to form the D enantiomer, or vice versa. An alanine racemase detected in *Medicago sativa* (Alfalfa) seedlings was the first such enzyme to be established in plants. (22) A serine racemase gene was recently identified and characterized in *Arabidopsis thaliana*. (23) The biological function of amino acid

racemases has not been determined in plants. Some bacterial racemases have been determined to be involved in the synthesis of peptidoglycans or poly-γ-glutamate, neither compound having an identified role in plants. (24)

In plants, it is unclear why a biosynthetic pathway for D-amino acids exists. Little is known about what, if any function D-amino acids serve. They are unusable as protein building blocks, and are not subject to any known enzymatic activity that could release the amine group as a usable form of nitrogen. (25) Plant cells conjugate limited amounts of free D-amino acids into N-malonyl and N-acetyl derivatives, which are then compartmentalized in the vacuole. (26) Since some D-amino acids are known to bring about negative effects on plant growth and development (27), compartmentalization may be a means to offset toxicity. In any case, the existence of a mechanism to isolate D-amino acids suggests to us that plants are unable to make use of any D enantiomer.

It has been shown that *Arabidopsis thaliana* seeds sown on media containing 3mM D-serine or 3mM D-alanine arrest development shortly after germination. (19) Several other plant species have demonstrated D-serine sensitivity at similar concentrations. (28) D-amino acids may interfere with endogenous protein synthesis, but the mechanism of their toxicity has not yet been elucidated. It has been hypothesized that D-serine competes with endogenous β-alanine in the synthesis of pantothenic acid (29) In an earlier study, however, incubation with exogenous β-alanine and pantothenic acid failed to rescue wild type *A. thaliana* seedlings from D-serine pressure. (19)

D-Amino Acid Oxidase. The oxidative deamination of D-amino acids is a FAD-dependent substitution of the amine group for a ketone group. In a DAAO catalyzed reaction, the D-amino acid is converted to an imino acid intermediate, reducing FAD in

the process. FAD is re-oxidized by molecular oxygen, releasing hydrogen peroxide. Subsequent hydrolysis of the imino acid forms an α-keto acid along with ammonia. (30) **(Fig.1)** The enzymatic activity of the DAAO flavoenzyme is highly specific to the D-configuration, and favors neutral amino acids. All non-plant organisms exhibit DAAO activity, which may play a role in detoxifying endogenous or environmental D-amino acids. (31)

Absent DAAO activity, plants may not be able to metabolize D-amino acids beyond a certain threshold. The basis for the varying phytotoxicity of different D-amino acids and their oxidative deamination products is unclear. (32) Catastrophic effects on plant development are observed in the presence of excess D-alanine and D-serine, and not their corresponding α-keto acids. (19) In contrast, D-valine and D-isoleucine have minimal effects on plant development, but are oxidatively deaminated into phytotoxic compounds. These compounds, 3-methyl-2-oxopentanoate and 3-methyl-2-oxobutanoate, completely inhibit plant growth at respective concentrations of 1mM and 5mM. (19) This dichotomy forms the basis of conditional selection using a plant oxidative deaminase transgene.

D-amino acid oxidase activity was first conferred to plants by the *dao1* gene from *Rhodotorula gracilis.* D-alanine and D-serine tolerance was observed in *Arabidopsis thaliana* plants expressing *dao1* from the CaMV 35S promoter. Enzymatic *rgDAAO* activity was quantified by incubating crude protein extracts from transgenic plants with D-alanine, and assaying for increased absorbance associated with its conversion to pyruvate. Although a range of *dao1* expression levels was established, phenotypes of the corresponding plants were uniform, indicating complete D-alanine and D-serine tolerance

irrespective of protein quantity. The *dao1* plants proved amenable to conditional selection as well, dying at levels of D-valine and D-isoleucine that were non-detrimental to the wild-type controls. (19)

A second selection scheme using D-amino acids is based on D-serine ammonia lyase activity conferred by the *dsdA* gene of *Escherichia coli.* DSDA activity targets D-serine with a higher specificity than DAAO and, with the exception of D-threonine, does not have a catalytic effect on any other D-amino acid. (33) The *dsdA* gene is practical for positive selection in conjunction with D-serine, but its specificity precludes its use for conditional selection. (32) The first application of *dsdA* to biotechnology was as a bacterial transformation marker, using strains of *E.coli* with deficient DSDA activity. (34) D-serine tolerance was later established in *A.thaliana* seedlings carrying the *dsdA* transgene. (32)

Overview. The objective of our study was to determine whether a non-herbicidal, non-antibiotic compound could efficiently provide selection in conjunction with a novel selectable marker gene. A DAAO gene from *Schizosaccharomyces pombe* has not previously been identified or characterized. Our goal was to determine whether a putative *S.pombe* sequence identified through homology was indeed a D-amino acid oxidase protein and furthermore, whether it could express DAAO heterologously at a degree sufficient to confer resistance to phytotoxic D-amino acids.

We transformed *Arabidopsis thaliana plants* with a modified D-amino acid oxidase gene from *S.pombe* and examined phenotypes of subsequent transgenic plants in the presence of D-alanine and D-serine. Our results show that D-alanine exerts selective pressure on wild type plants equal to other commonly used selection substrates, and that

sufficient activity is expressed from sp*DAAO* to confer tolerance to plants carrying the gene. Homologous DAAO genes are ubiquitous in all non-plant organisms, as are the D-amino acid substrates themselves. This may alleviate some of the aforementioned issues with plant selectable markers. We summarize the advantages of the DAAO system here:

- *Broad spectrum activity*: Plants contain very low amounts of all D-amino acids. Some, such as D-alanine and D-serine, have been shown to inhibit plant development when present in excess quantities. Although their mechanism of toxicity has not been verified, preliminary evidence indicates that D-amino acids may affect endogenous protein metabolism in all plant species. (19) As DAAO activity has never been established in plants, this draws attention to the potential that D-amino acids may have as broad-spectrum selective agents or herbicides.
- *Benign selective agent*: Pathways for D-amino acid oxidative deamination, as well as the presence of D-amino acids themselves, have been identified in a wide variety of prokaryotes and eukaryotes, including humans. (17) This suggests to us that unlike antibiotic selection agents, D-amino acids and their by-products influence plant phenotype with less risk of adverse environmental outcomes or effects on human health.
- *Conditional selection*: *spDAAO* is a conditional marker, allowing for positive or negative selection depending on which D-amino acid substrate is used. While D-alanine and D-serine have demonstrated broad-spectrum phytoxicity, their oxidative deaminase products are more benevolent to the plant. Conversely, there are other D-amino acids such as D-valine and D-isoleucine that do not affect plant growth, but are converted by the same reaction into inhibitory compounds. (19)

Therefore, when used alongside marker-removal systems such as CRE/*LoxP*, plants containing a *DAAO* marker gene as well as subsequent marker-free plants from which the *DAAO* is absent would both be identifiable using positive selection. (35)

Results

Characterization of the S.pombe DAAO. A protein BLAST query of the *Rhodotorula gracilis* DAAO amino acid sequence yields a putative *Schizosaccharomyces pombe* DAAO protein with 32% amino acid identity. (36) There have been no previous attempts to produce a recombinant DAAO enzyme from *S.pombe.* The *sp*DAAO amino acid sequence contains 348 residues, with unexpectedly low homology to any other known or hypothetical DAAO sequences. *sp*DAAO has 43% similarity to *Aspergillus* species, and 36% similarity to *Trigonopsis variabilis.*

An amino acid sequence alignment of *sp*DAAO with representative homologous proteins was performed using the ClustalW program. (37) The putative spDAAO was shown to cluster most closely with other well-characterized fungal DAAO proteins. **(Fig.2A)** Putative domains were annotated based on homology with certain amino acid sequences of the *T.variabilis* DAAO enzyme, which has been characterized in previous studies using X-ray structural analysis. (38) (**Fig.2B**) A conserved N-terminal sequence reflects a dinucleotide-binding motif (39) known as a Rossmann fold, which places this protein in the glutathione reductase family. (40) Rossmann folds represent FAD binding domains in all flavoenzymes. (41)

45% of the conserved residues fall into three clusters representing catalytic portions of the active site. Previous studies have revealed low DAAO homology among D-amino acid binding sites, which may account for differences in substrate specificity among species. (42)

The *sp*DAAO sequence does not include a conserved area near the C-terminus that serves as a peroxisomal targeting signal in many homologous flavoenzymes. (43)

This is consistent with the absence of a peroxisome in *S.pombe.* (44) The location of a heterologous *sp*DAAO protein in the transgenic plant is unclear, and it remains to be seen whether the possible accumulation of the hydrogen peroxide by-product could affect the plant. This would be less of a concern if marker gene removal were performed.

Establishing D-alanine and D-serine as selection substrates. Wild type *Arabidopsis thaliana* (Col ecotype) seeds were germinated on 0.5X MS media containing a dilution series of 3mM, 5mM, and 10mM D-serine (Sigma S4250) or D-alanine (Sigma A7737). Seedlings were screened after 10 days to determine the degree of growth inhibition on 3mM D-serine and 3mM D-alanine relative to a positive control plate with no selection. (**Fig.3A-3C**) Identical effects were observed at higher concentrations of both substrates.

Seedlings on the control plate were green and exhibited normal development of the cotyledon and first leaf pair. Under pressure from D-serine, seedlings germinated and turned green, but exhibited slower growth and did not develop a first pair of leaves. In the presence of D-alanine, growth was completely arrested shortly after germination, with minimal greening.

Wild type seedlings grown on plain 0.5X MS media were transferred to media containing 3mM D-serine and 3mM D-alanine after 8 days. Seedlings were screened on day 15. (**Fig.3D-3F**) On D-serine, seedlings exhibited signs of stress, such as anthocyanin accumulation and early flowering. Seedlings on D-alanine arrested growth and bleached within one week.

***Vectors.* (Figs. 4A-4D)** The three vectors generated in this study contain the sp*DAAO* gene driven by a nopaline synthase (*nos*) promoter. pDAAO1 also contains an

hptII cassette conferring hygromycin resistance. The pDAAO2 vector contains the pUC18 polycloning site along with sp*DAAO* as the exclusive selectable marker. pDAAO3 contains the *spDAAO* cassette, a GUSplus cassette, and the full polycloning site from pUC18. Wild type *Arabidopsis thaliana* plants (Columbia ecotype) were transformed using the floral dip method. (45)

Selection analysis. To screen for pDAAO1 transformation, approximately 30,000 surface-sterilized T1 seeds were sown on solid 0.5X MS medium containing 20μg/mL hygromycin. After 48 hours at 4^{O}C, seeds were placed in the dark at room temperature for three days then examined for hypocotyl elongation. **(Fig. 5A)** Over 100 hygromycin resistant seedlings were identified in this manner; 72 seedlings were transferred to soil.

pDAAO2 T1 seeds were collected for further screening.

To test primary selection, we used approximately 30,000 surface-sterilized T1 seeds from the pDAAO3 dipped plants. Seeds were sown onto plates containing solid 0.5X MS media plus 5mM D-serine or 5mM D-alanine. After 48 hours at 4^{O}C, seeds were placed at 22^{O}C under a 16h / 8h photoperiod. Screening was performed after 10 days. We searched for seedlings exhibiting wild-type growth under selective pressure.

Ease of selection on D-alanine was facilitated by the earlier developmental arrest, relative to D-serine, of untransformed seedlings. **(Fig. 5B)** Putative transformants on D-serine were camouflaged against their surroundings due to the green color of untransformed seedlings. The determining factor for selection was formation of the first leaf pair. **(Fig. 5C)**

15 transgenic T1 seedlings exhibiting tolerance to D-serine or D-alanine were selected for GUS staining; 72 others (36 of each) were moved to soil.

GUS staining. pDAAO3 plant tissue was subjected to a GUS staining treatment to confirm insertion of the sp*DAAO* cassette. Of the 15 pDAAO3 primary transformants, six exhibited blue staining after one hour, while the remainder exhibited staining after an overnight incubation. **(Fig. 6)** We assayed all of the 72 plants on soil in the same manner, subjecting leaf and inflorescence pieces to incubation with X-Gluc. 22 lines exhibited blue staining after one hour, with a total of 65 showing color following overnight incubation. There was no apparent staining in 7 of the lines. Further testing of T2 seed progeny will determine whether the sp*DAAO* cassette is present in these plants.

Titanium Assay. Measurements of endogenous hydrogen peroxide levels were taken to detect oxidative deaminase activity conferred from the heterologous *spDAAO* gene. Previous studies have shown that hydrogen peroxide reacts with titanium to form a complex that is yellow in color. The intensity of spectophotometric absorption at 415 nm is proportional to the amount of titanium(IV)-peroxide present in solution. (46)

Leaf tissue was taken from 9 soil-grown adult plants expressing the pDAAO3 construct. These were a subset of the 22 lines exhibiting GUS staining after one hour. A wild type (Col ecotype) control was also tested. Tissue was homogenized in 5% trichloroacetic acid, and subjected to centrifugation. Supernatants were removed to new tubes with two drops of titanium reagent added to each. Absorbency was measured at 415 nm, with peroxide levels determined according to a standard curve. **(Table 1)** Relative to the control, color change was immediately visible in the test solutions, indicating the presence of hydrogen peroxide at significantly higher levels. **(Fig.7)** It is important to note that the plants used in this experiment were removed from media containing D-amino acids one month prior to testing. Therefore, we hypothesize this

result as a direct function of oxidative deaminase activity on naturally occurring levels of D-amino acids in the plant, or on alternative endogenous substrates.

Discussion

In this study, we tested the feasibility of using the *DAAO* gene from *S.pombe* as a plant selectable marker. We have shown that sp*DAAO* confers sufficient oxidative deaminase activity from the plant to allow tolerance to D-alanine and D-serine. Transgenic *spDAAO* plants grow with apparently normal wild-type phenotypes in the presence of selection. In contrast with findings from a previous study (19), we found that D-serine does not affect plant development to a degree suitable for selection purposes. Although plants under D-serine pressure were clearly stunted, we observed more greening than expected. The aforementioned study tested an identical (Columbia) *A.thaliana* ecotype on 0.5X MS media with sucrose added at a 0.5% concentration. We did not include sucrose in our growth medium. Therefore, this discrepancy in phenotype cannot be accounted for by native serine racemase activity, and is more likely a function of sucrose, or alternate sources of D-serine.

Future studies involving *Arabidopsis* transformation will utilize D-alanine as a selective substrate, as it proves most efficient in negatively selecting against untransformed plants. Vectors pDAAO2 and pDAAO3 contain a polycloning site to facilitate insertion of any future genes of interest.

Adult transgenic T1 plants had normal phenotypes and were fertile, indicating that the insertion and expression of the sp*DAAO* cassettes is not likely to create unwanted side effects. One cause for concern was whether the associated increase in peroxide levels would prove detrimental to plant health. Unlike many yeasts, including *R.gracilis*, *S.pombe* does not possess a peroxisome. Correspondingly, the native spDAAO protein sequence does not include a conserved peroxisomal targeting sequence found at the C-

terminal end of many homologs. Although the location of the recombinant *sp*DAAO protein remains unknown, this study reveals that its present level of expression does not harm the plant.

From the titanium assay, it is clear that peroxide levels were substantially higher in transgenic sp*DAAO* plants. However, future tests will specifically address whether this is a direct result of DAAO activity on D-amino acids. There are a number of ways to accomplish this. The titanium assay could be repeated to compare peroxide content in transgenic plants taken directly from varying levels of D-alanine selection. We would expect to observe an increase in peroxide levels proportional to about of D-alanine substrate available for oxidative deaminase activity from spDAAO. We could also assay directly for the production of α-keto acids in transgenic plants using HPLC or by measuring the associated absorbance in a soluble protein extract incubated with D-amino acids.

Further characterization of the DAAO selectable marker gene system will involve comparison of wild-type and sp*DAAO* plants in the presence of D-valine and D-isoleucine with the goal of elucidating whether positive selection for marker gene absence is feasible using sp*DAAO*. This class of conditional selection would have particular application to marker gene removal systems where site-specific recombinases mediate the removal of sequences flanked by distinct excision sites. Removable marker gene systems allow for the repetitive use of the same marker when introducing multiple genes into plants, and aid in the production of marker-free transgenic plants. (47) Following excision, however, it is difficult to efficiently confirm marker gene removal because of the requirement for two separate marker genes to identify initial transformants

vs. subsequent marker-free progeny. A conditional selection scheme would make it possible to positively select for both the presence and absence of a marker gene. (32) A single marker gene conferring different effects to the plant in the presence of varying selective substrates would facilitate this.

Materials and Methods

Luria-Bertani broth (EMD 1.10285) was used to grow *Escherichia coli* during the cloning steps. Growth medium for *Agrobacterium tumefaciens* is described in the plant transformation protocol**. (Appendix 1)** Murashige & Skoog medium (RPI M10200) was used as the germination substrate for plants. MS media was prepared at 0.5X concentration with pH adjusted to 5.8 using potassium hydroxide, and solidified with 0.8% bacteriological grade agar (Ambresco J637).

Vector construction. **(Appendix 2)** A BLAST search (36) was used to identify a putative D-amino acid oxidative deaminase open reading frame in the *S.pombe* sequence, based on homology to the *Rhodotorula gracilis dao1* protein. Genomic *S.pombe* DNA was used as PCR template and amplified using primers 5'-ccgtgatcaatataaaca**ATG**ACTAAGGAAAATAAGCCAAGAG-3' and 5'-gtgccatggctcgagacctaagccaattttGATTTTAGGAAGAGC-3'. The first primer appended a BclI site (tgatca) to the 5' end, while the second attached NcoI (ccatgg) and XhoI (ctcgag) sites to the 3' end.

A pGEM-T vector (Promega) containing the *nos* promoter was used as a backbone for cloning. The PCR fragment was subcloned as a BclI/NcoI fragment into the BamHI/NcoI - digested pGEM-T vector, downstream of Pnos. p*Nos*Pro-DAAO was transformed into *E.coli* (DH5α strain). The transformation mix was plated on solid LB media containing ampicillin (100μg/mL) for selection. Plasmid DNA was prepared from ampicillin resistant colonies grown overnight at 37^{O}Cin 3mL liquid LB culture. The p*Nos*Pro-DAAO vector was subjected to restriction analysis to verify the precise entry of the *DAAO* coding region.

Sequencing was performed to confirm the *spDAAO* sequence, using standard M13 and nos primers (48) as well as the following specific DAAO primers:
5'-CTACGGACATAAGGCCACCACCTT-3' and
5'-AGCCCAGAGACCAGTGCAATTAAA-3'.

The (Pnos)-(DAAO) fragment was isolated from pGEM-T with NcoI/SalI and ligated into the corresponding restriction enzyme sites in the polycloning site of pCAMBIA1390, upstream of a *nos* terminator to complete the (Pnos)-(sp*DAAO*)-(Tnos) construct. The resulting vector also contains a (double P35S)-(*hptII*)-(T35S) cassette, conferring plant hygromycin resistance. The recombinant vector was named pDAAO1.

The SphI/XhoI fragment from pDAAO1, containing (Pnos)-(sp*DAAO*), was cloned into BstXI/XhoI-digested pCAMBIA2300 to replace the (double P35S)-(*nptII*) portion of its kanamycin resistance gene. The resulting vector contains the (Pnos)-(*spDAAO*)-(T35S) cassette along with the pUC18 polycloning site in its T-DNA region. The recombinant vector was named pDAAO2.

The SphI/XhoI fragment from pDAAO1, containing (Pnos)-(sp*DAAO*), was cloned into BstXI/XhoI-digested pCAMBIA1305.1 to replace the (P35S)-(*hptII*) portion of its hygromycin resistance gene. The resulting vector contains the (Pnos)-(*spDAAO*)-(T35S) and (double P35S)-(*GUS)*-(Tnos) cassettes in its T-DNA region along with the pUC18 polycloning site. The recombinant vector was named pDAAO3.

Successful cloning of the three binary vectors was confirmed using restriction analysis. The above cloning steps were performed using standard restriction endonuclease and T4 ligase treatments, along with a heat-shock bacterial transformation protocol. (49)

GUS staining. We prepared two buffer solutions consisting of 0.1M Sodium Phosphate (Sigma SX0710-1), 10mM EDTA (EM Science, 9530), 10% Triton X (BioRad, 161-0407), 0.5mM Potassium Ferrocyanide (EM Science, PX1460-1), and 0.5mM Potassium Ferricyanide (EM Science, PX-1455-2) in water. An X-Gluc stock solution was prepared using powdered X-Gluc (Gold Biotechnology, G1281C1) dissolved in DMSO (Fisher Scientific, D136-1), and added to one of the buffer solutions to give a final concentration of 2mM X-Gluc.

Plant tissue was placed in 90% acetone solution for 10 minutes at -20^{O}C. The samples were subsequently rinsed with water then washed in buffer without X-gluc. Samples were next placed into buffer with X-gluc, subjected to vacuum infiltration for two minutes, and kept in buffer for incubation at 37^{O}C in darkness. Samples were checked for staining after one hour and once more following overnight incubation.

***Titanium Assay*.** Plant tissue was homogenized in 5% Trichloroacetic acid (Fisher, 76-03-9), and centrifuged at maximum speed for one minute. Two drops of titanium reagent were added to assay for level of hydrogen peroxide in the supernatant. Titanium reagent is a 20% solution of Titanic tetrachloride (Sigma, 7550-45-0) in concentrated HCl (Fisher Scientific, A144-212). In order to quantify amounts in this experiment, a standard curve was prepared using hydrogen peroxide stock solution diluted to 2, 1, 0.3, 0.1, 0.03, and 0.01 mM in 5% Trichloroacetic acid with two drops of titanium reagent added per 2mL H_2O_2 solution. (50)

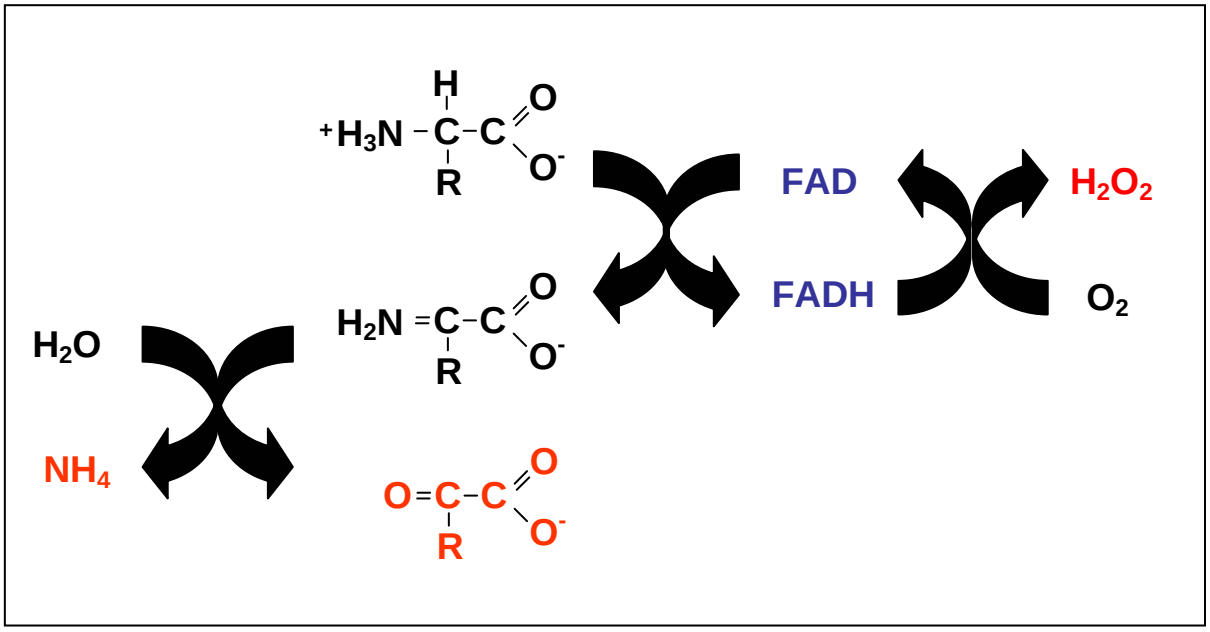

Figure 1. The oxidative deamination of D-amino acids. (31) In the DAAO catalyzed reaction, the D-amino acid is converted into an imino acid intermediate, reducing FAD in the process. FAD is reoxidized by molecular oxygen, releasing hydrogen peroxide. Subsequent hydrolysis of the imino acid forms an α-keto acid along with ammonia.

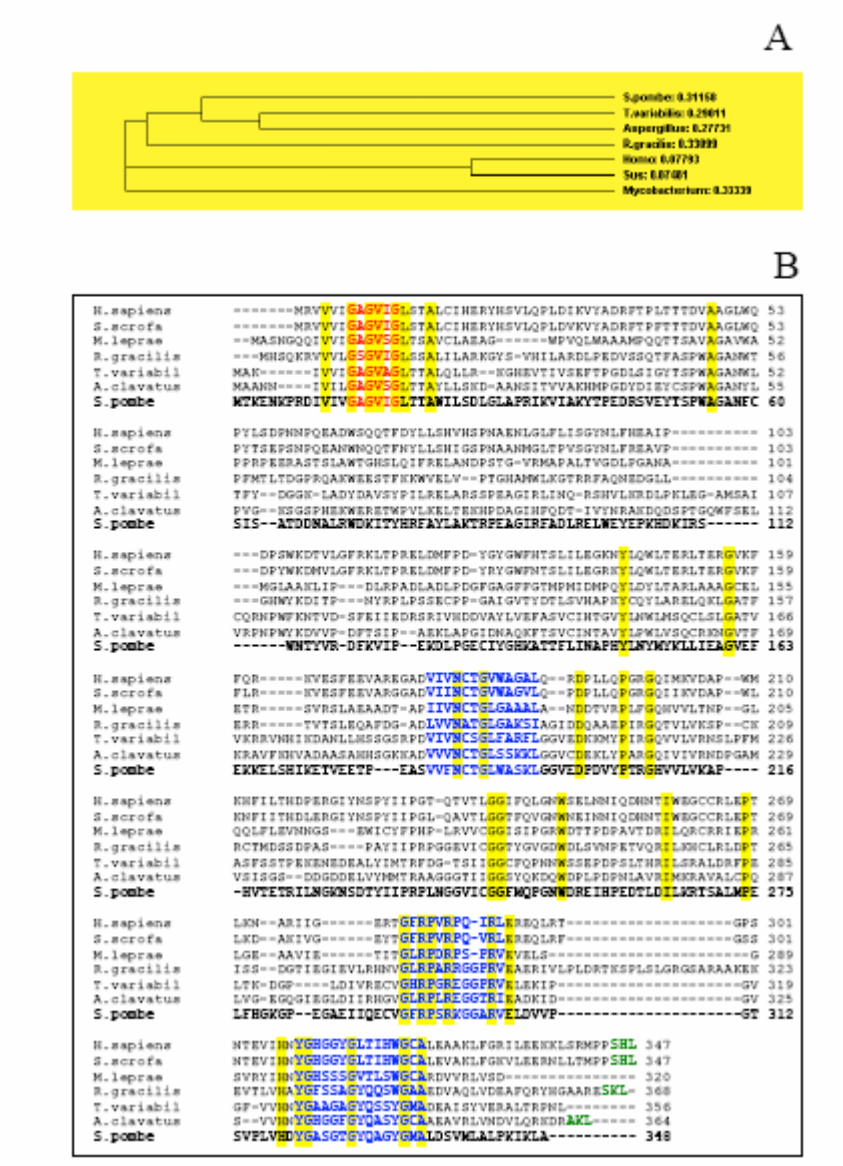

Figure 2. Characterization of the *S.pombe* DAAO. The evolutionary relationship of DAAO homologs (A) was obtained from a ClustalW alignment (36) of our spDAAO sequence against well-characterized bacterial, fungal and mammalian DAAO enzymes. The putative S.pombe sequence clusters most closely with other fungal DAAO proteins. The 348 residue sequence of spDAAO is shown in comparison to representative known homologs. (B) Amino acid residues conserved across kingdoms are highlighted in yellow. Residues of FAD-binding domain are colored red, while the three clusters in blue represent active portions of the catalytic site. (38)

Figure 3. Establishing D-alanine and D-serine as selection substrates. Seeds on the control plate germinated normally, with green color and ordinary development of the cotyledon and first leaf pair. (A) In the presence of D-alanine, growth was completely arrested shortly after germination. (B) Under pressure from D-serine, seedlings germinated and turned green, but exhibited slower growth and did not develop the first rosette. 8-day old seedlings were transferred to selection, and examined on day 15. Compared with the control (D), seedlings on D-alanine arrested growth and bleached out within one week. (E) Seedlings on D-serine exhibited signs of stress, such as anthocyanin accumulation and early flowering. (F)

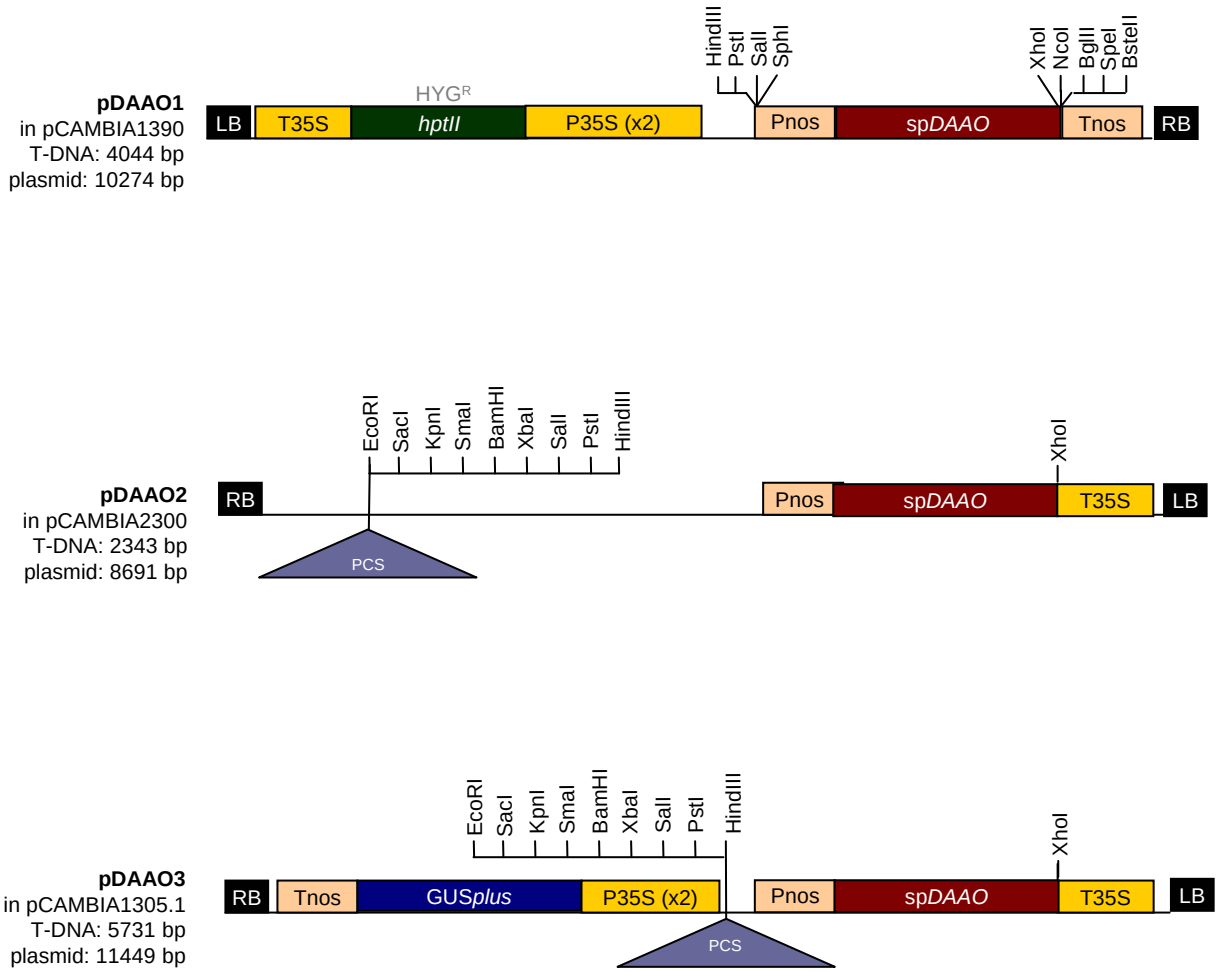

Figure 4. Vectors. The three binary vectors generated in this study contain the sp*DAAO* driven by a nopaline synthase promoter. Additionally, pDAAO1 contains an *hptII* cassette conferring resistance to hygromycin. pDAAO3 contains a GUS construct to facilitate X-gluc staining. pDAAO2 and pDAAO3 contain the pUC18 polycloning site to facilitate insertion of future genes of interest.

Figure 5. Selection Analysis. To test for pDAAO1 transformation, T1 seeds were sown on solid 0.5X MS medium containing 20 μg/mL hygromycin, and examined for hypocotyl elongation. (A) Ease of primary selection on D-alanine was facilitated by the earlier developmental arrest, relative to D-serine, of untransformed seedlings. (B) Primary transformants on D-serine were camouflaged against their surroundings due to the greening of untransformed seedlings. The determining factor for selection was formation of the first rosette.

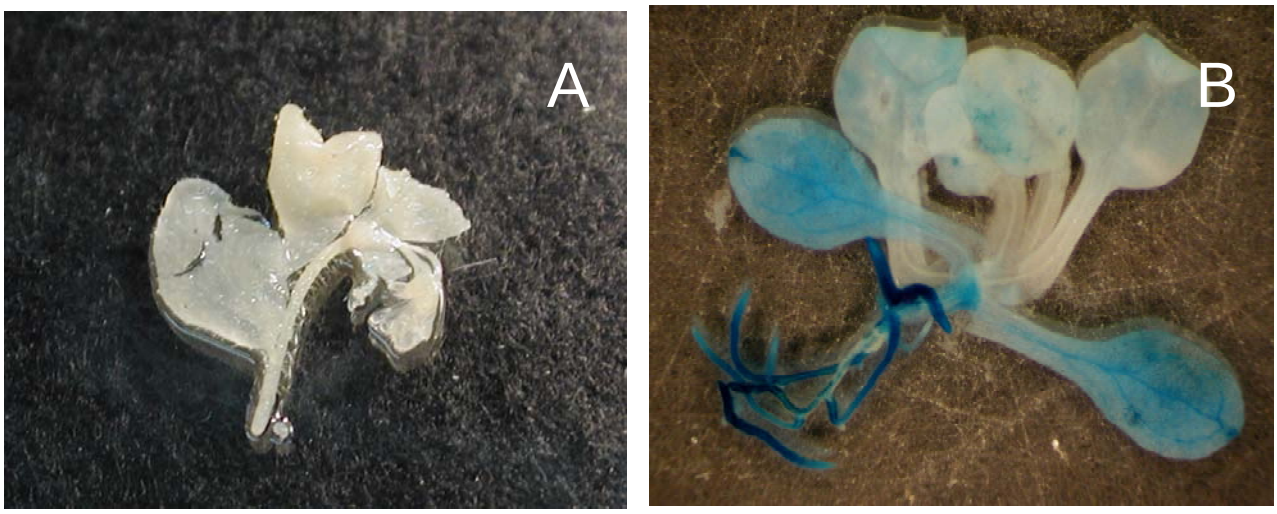

Figure 6. GUS staining. pDAAO3 plant tissue was subjected to a GUS staining treatment to confirm insertion of the sp*DAAO* cassette. 15 seedlings were selected from primary transformation plates for staining. Compared with the negative wild-type control (A), six seedlings exhibited blue staining after one hour, with the remainder staining blue after an overnight incubation. (B)

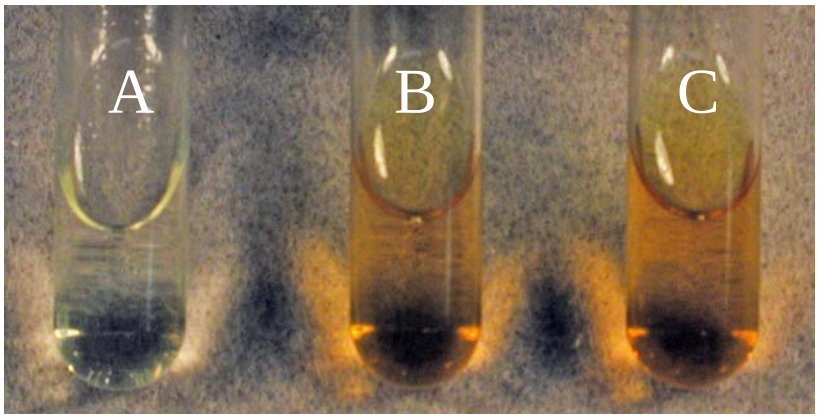

Figure 7. Visible color change in titanium test solutions. Relative to the control, (A) color change was immediately visible in the test solutions. Samples #5 (B) and #9 (C) from Table 1 are shown here. Yellow color indicates the presence of hydrogen peroxide at significantly higher levels. We hypothesize this result as a direct function of oxidative deaminase activity on naturally occurring levels of D-amino acids in the plant, or on alternative endogenous substrates.

#	OD (415nm)	uM /mL
1	0.18	0.29
2	1.60	2.61
3	1.24	2.02
4	1.25	2.04
5	2.31	3.76
6	1.41	2.30
7	1.25	2.04
8	1.85	3.01
9	2.09	3.40
10	1.29	2.10

Table 1. Quantifying peroxide levels in DAAO plants. Based on the standard curve, a reading of OD 0.0614 corresponds with 0.1mM of hydrogen peroxide in 1mL solvent. H_2O_2 amounts were quantified by dividing OD of each sample by 0.0614, and multiplying by 0.1mM to obtain µM/mL. Sample 1 is a wild type control.

Appendix 1. Plant Transformation Protocol

A. Reagents

1. **Agro LB**

   in **500 mL H_2O**

   autoclaved

Tryptone	**5.0 g**
Yeast Extract	**2.5 g**
NaCl	**2.5 g**

Add for solid media:

Agar	**6.0 g**

2. **YM media**

   in **1L H_2O**

   autoclaved

Mannitol	**10.0 g**
K_2HPO_4	**0.5 g**
Yeast Extract	**0.4 g**
$MgSO_4$	**0.2 g**
NaCl	**0.1 g**

3. **IM (inoculation media)**

   in **1L H_2O**

   Freshly made before each use

Sucrose	**50 g**
$MgCl_2$ (1M)	**10 mL**
Silwet	**50 *u*L**

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B. Transforming Agrobacterium (Electroporation)

1. Agrobacterium strain, 100 uL stocks, can be found in the -80°C freezer.
2. Combine each tube of **100uL Agro** with **500 uL *cold sterile* H_2O**
3. Aliquot **100 uL** of ***diluted* Agro** into sterile cuvettes, **on ice**.
4. Calculate amount of DNA to add:
    a. Amount should be between **50-500 ng**
    b. DNA must be Qiagen-purified
    c. Quantify your miniprep, then fill in the following table accordingly:

Sample ID	Sample Conc.	Volume added	Amount of DNA
#	*ug/mL* (= *ng/uL*)	*uL*	*ng*

5. BioRad E.coli pulser:
    a. Press <**SET VOLTS**>
    b. Charge up capacitor to the maximum **2.5 kV**
    c. **Add DNA** to the 100uL Agro in cuvette and mix well
    d. Insert cuvette into machine
    e. Press BOTH <**PULSE**> buttons until you hear the beep
    f. IMMEDIATELY add **1 mL YM medium**
    g. Leave at room temp for 2+ hours, then plate on solid AgroLB+selection

6. Incubate plates at 28°C for up to two days for visible colonies
7. Streak single colony onto new plate and incubate at 28°C to obtain more single colonies

8. For each clone, inoculate one of these single colonies into **3-5 mL** *liquid* **AgroLB** +***selection*** and spin overnight at 28°C

9. Once they've grown:
    a. Make a frozen stock: Combine **500uL** each**: 50% Glycerol** + **Agro** culture
    b. Inoculate 2 mL of **Agro** culture into **200 - 250 mL** *liquid* **AgroLB** +***selection*** and shake overnight at 28°C

C. Transforming Arabidopsis (The Floral Dip)

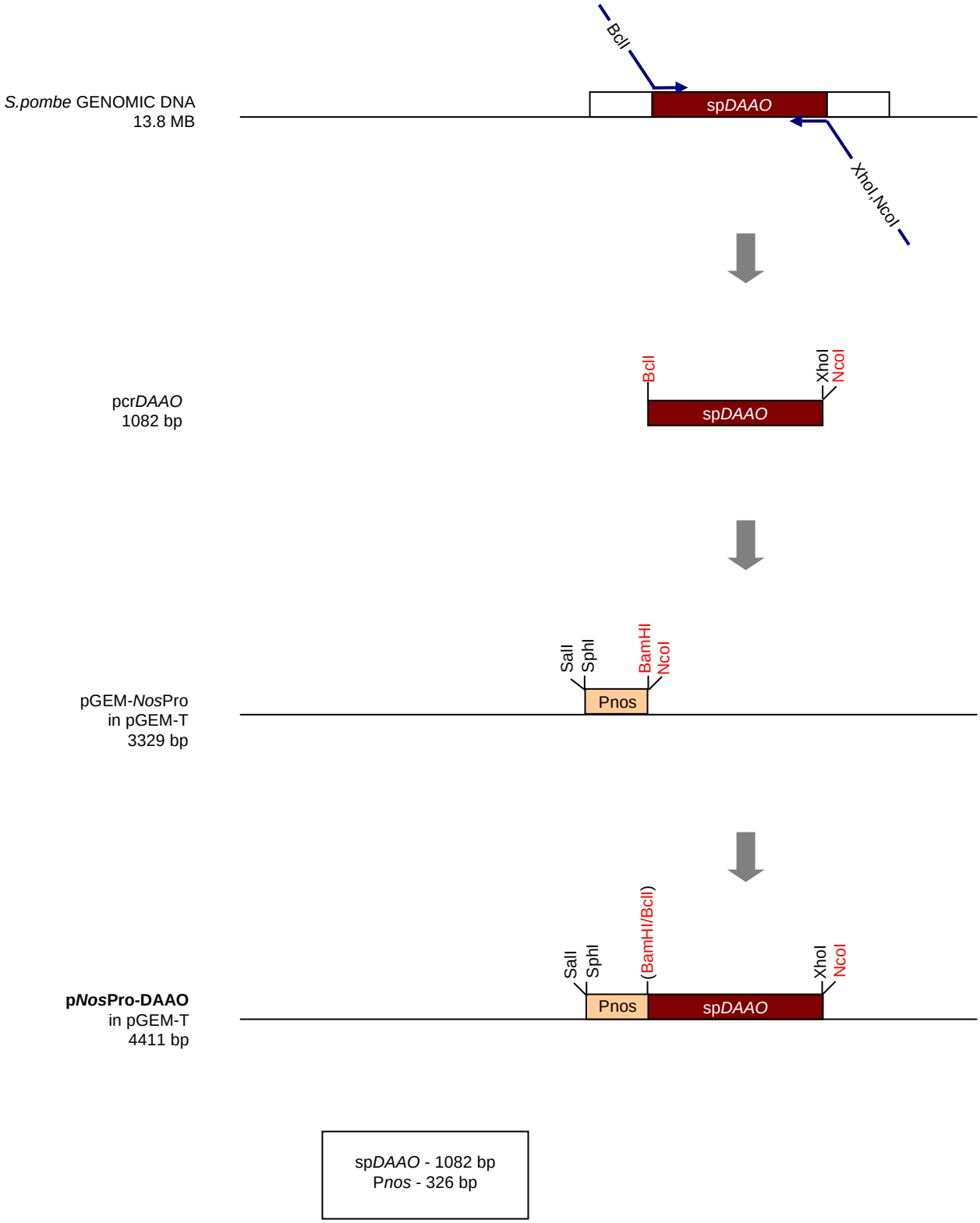
1. Transfer your 200-250mL of inoculum into a centrifuge tube and spin down cells (**20 min, 4000 rpm, 28°C**)

2. Remove supernatant and resuspend Agro pellet in **400-500 mL IM medium** (2x volume)

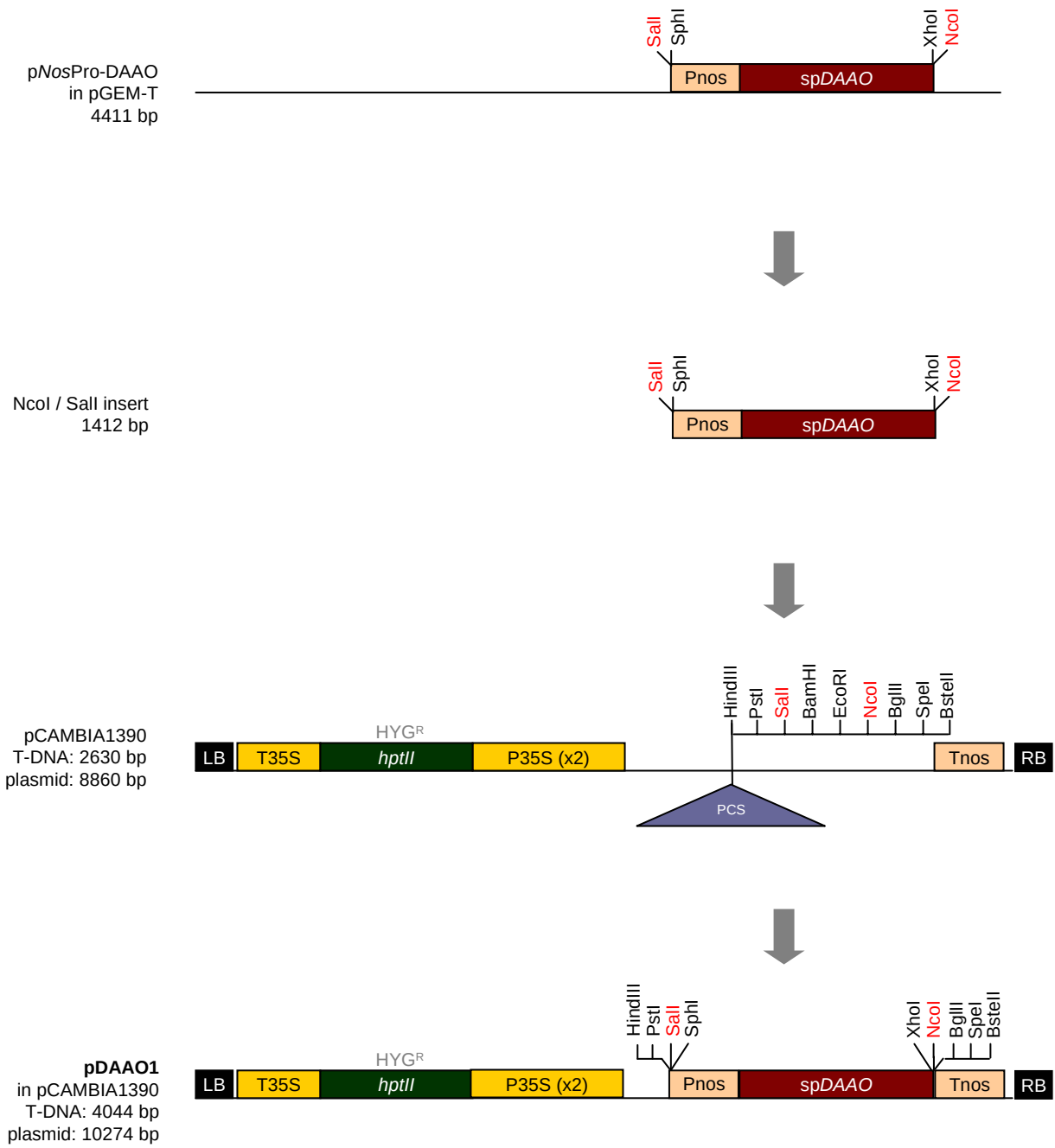
3. Pour **IM/Agro** solution into a clean autoclaved container (empty gel boxes work nicely)

4. Dip your Arabidopsis plants:
   - **a.** Plants should be recently watered, so that soil doesn't take up all the Agro
   - **b.** Make sure all the axillary buds are submerged for 15-30 seconds
   - **c.** Plant should be gently swirled around while submerged

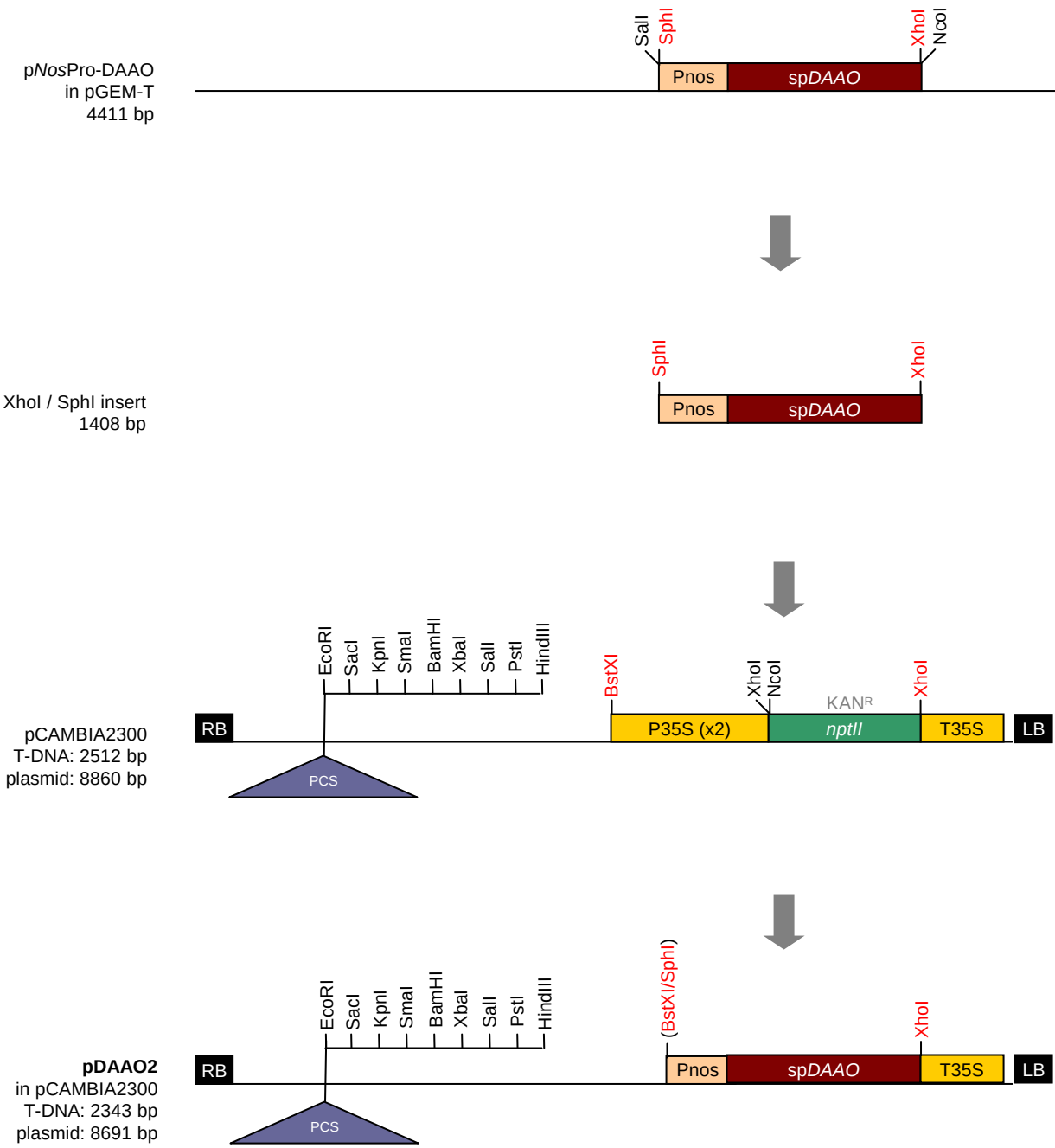
5. After dipping, plants are placed in the growth chamber: lay them on their sides and cover overnight. They can be placed upright the next day.



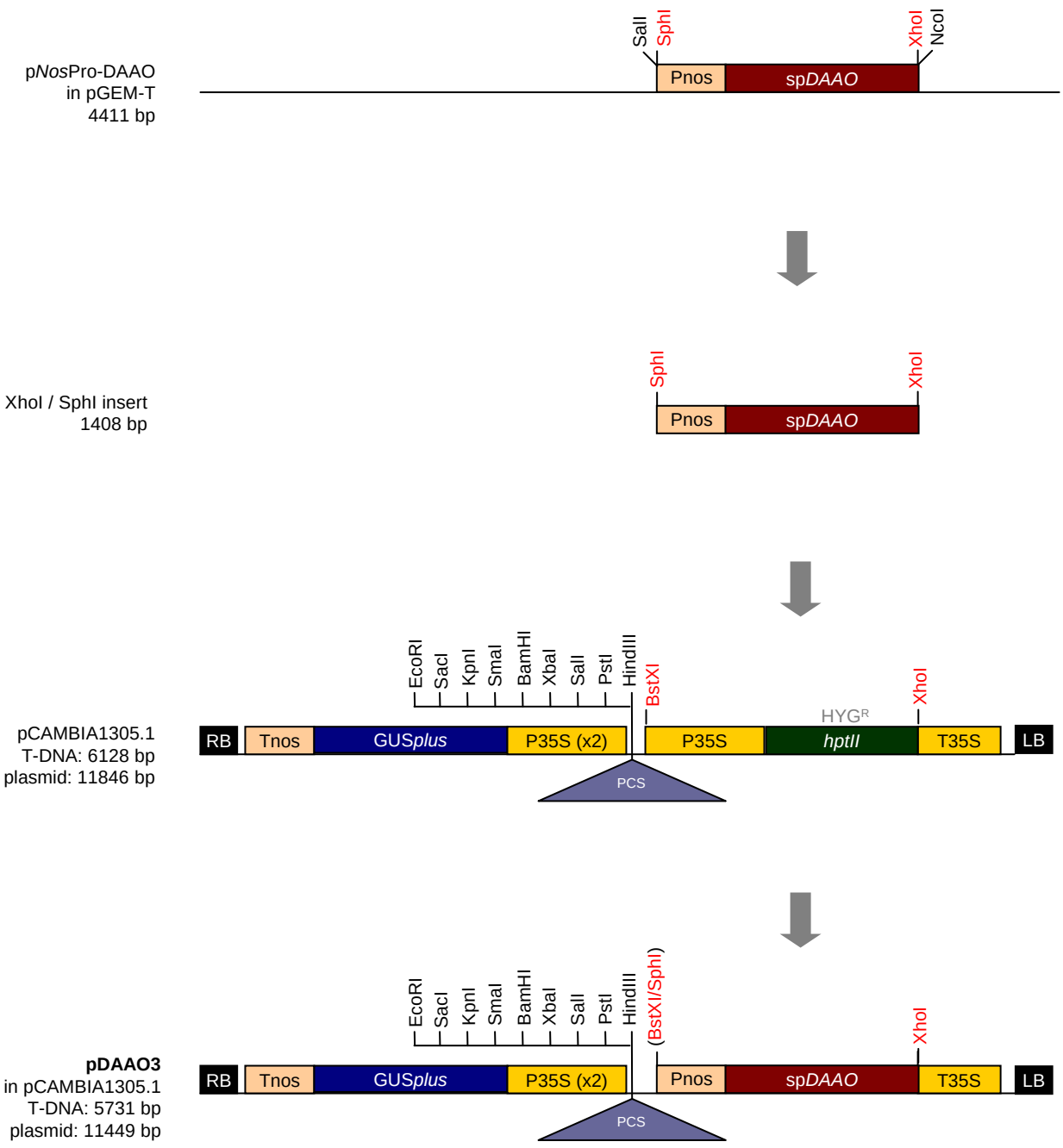
Engineering the P*nos-DAAO* construct



Construction of pDAAO1



Construction of pDAAO2



sp*DAAO* - 1082 bp	P35S - 780 bp
hptII - 1025 bp	T35S - 208 bp
P*nos* - 326 bp	LB - 25 bp
T*nos* - 252 bp	RB - 25 bp
PCS - 56 bp	GUS*plus* - 2052 bp

Construction of pDAAO3

Appendix 3. Mammalian and Microbial DAAO activity

D-amino acid oxidase (DAAO) activity is ubiquitous in all kingdoms except plants. The enzyme has been identified and isolated in fish, reptiles, amphibians, insects, birds, and mammals. (17)

DAAO was first isolated from mammalian tissue in 1935. (17) It has attracted attention for its practical application in the synthesis of cephalosporin antibiotics, which bind and crosslink peptiodglycan precursors, preventing formation of the bacterial cell wall. (51) Oxidative deamination of Cephalosporin C results in the formation of 7-aminocephalosporanic acid, which is used in the manufacture of several synthetic cephalosporins. (52)

Mammalian DAAO has been well characterized in a variety of organisms. The crystal structure of DAAO was first generated in the 1980s, using enzyme isolated from wild boar (*Sus scrofa*) kidney. (53) DAAO is a peroxisomal enzyme, which keeps the potentially toxic hydrogen peroxide by-product sequestered from the rest of the cell. (54) The presence of DAAO has been established in mammalian liver, kidney, and brain cells. Enzymatic activity has been shown to increase with D-amino acid content (55). Furthermore, malignant cells have lower levels of DAAO activity than their healthy counterparts. It is believed that oxidative deamination may play a role in detoxifying endogenous D-amino acids. (17)

In addition to the traditional DAAO, humans also retain a second oxidative deaminase enzyme specific to D-aspartate. The conversion of L-aspartate to D-aspartate is relatively high in humans, and is modulated by D-aspartate oxidase (DAPSO). The

exact role that D-aspartate plays is not clear, however increases in D-aspartate levels contained within cerebral white matter seem to correlate with aging. DAAO plays a role in the human nervous system by maintaining D-serine levels in the nervous system. D-serine is produced by a specific racemase in the brain (56), and interacts with N-methyl-D-aspartate (NMDA) receptors. (57) The activity of DAAO in the human brain is modulated by the G72 protein, also known as a D-amino acid oxidase activator (DAOA). Low G72 levels have been linked to the onset of schizophrenia. (58)

Microbial DAAO activity was first demonstrated in studies of the yeasts *Rhodotorula gracilis* and *Trigonopsis variabilis,* which displayed increased DAAO activity in the presence of D-alanine. (59) DAAO is also present in prokaryotes, as is evidenced by studies with *Mycobacterium tuberculosis, Streptomyces avermitilis,* and *Acinetobacteria* species. (60) Exogenous D-amino acids are usable as growth substrates in microorganisms thanks to oxidative deamination, which releases functional carbon and nitrogen. (29) Interest in DAAO has more recently expanded to microbial DAAOs because of their application to biotechnology. Higher quantities of chimeric DAAO protein can be extracted from *E.coli* expressing a *DAAO* gene with microbial origins. (61) DAAO activity has been also been established in microorganisms like algae, fungi, yeast and bacteria. (17)

Appendix 4. Static Test of *Lemna minor* Under Selective Growth Conditions

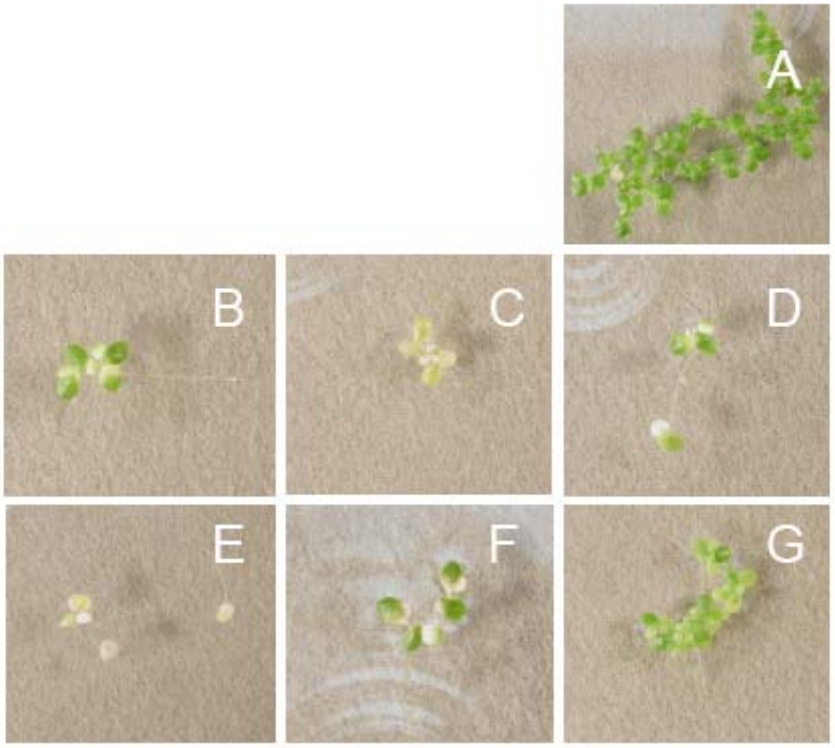
A growth assay using *Lemna minor* (Accession # UTCC492) was performed to test the phytotoxicity of several antibiotics and herbicides commonly used in the laboratory.

Lemna fronds were placed in flasks containing 0.5X Hoagland's No.2 Basal Salt Mixture (Sigma, H2395), with sucrose added at a concentration of 10g/L. We added varying concentrations of kanamycin, spectinomycin, gentamicin, PPT, D-alanine, and D-serine. Flasks were placed at 22^{O}C under a 16h/8h photoperiod.

Colonies were inspected over a two-week period for changes in growth and appearance. We assayed for number of total and chlorotic fronds on days 1, 3, 7, 10, and 14. Photographs taken on day 14 show the typical phenotype of *Lemna* colonies under pressure from the various selective substrates. **(Appendix Fig. 1)**

Each of the six phytotoxic compounds that we tested significantly reduced *Lemna* growth at every concentration. Final frond counts were all less than 10% of the control. Growth was immediately arrested at all concentrations of PPT, at gentamicin, 50 and 100 ug/mL, and at 10mM D-alanine. **(Appendix Table 1)**

A substantial percentage of fronds developed chlorosis in the presence of all six compounds. 100% chlorosis was achieved after 14 days under PPT and D-alanine pressure. **(Appendix Table 2)**



Appendix Figure 1. ***Lemna*** **phenotypes under selective pressure.** Relative to the control (A), fronds developed chlorosis under pressure from all six compounds: kanamycin (B), D-alanine (C), gentamicin (D), phosphinothricin (E), spectinomycin (F), and D-serine (G).

	12/17	12/20	12/24	12/27	12/31
Control 1	2	4	12	31	112
Control 2	4	9	31	87	208
Kan 5 ug/mL	4	4	6	7	8
Kan 50 ug/mL	4	4	7	7	7
Kan 100 ug/mL	5	8	8	9	10
Spec 10 ug/mL	4	8	10	14	15
Spec 100 ug/mL	5	8	9	9	9
Spec 200 ug/mL	4	5	7	8	8
Gent 5 ug/mL	4	5	7	7	7
Gent 50 ug/mL	4	4	4	4	--
Gent 100 ug/mL	4	4	4	4	4
PPT 2 mg/L	4	4	4	4	4
PPT 4 mg/L	4	4	4	4	4
PPT 10 mg/L	4	4	4	4	4
D-Ala 1 mM	4	4	6	6	6
D-Ala 3 mM	4	5	6	6	6
D-Ala 10 mM	4	4	4	4	4
D-Ser 1 mM	4	8	9	14	27
D-Ser 3 mM	4	5	9	10	16
D-Ser 10 mM	4	8	9	10	11

Appendix Table 1. Number of fronds. We measured growth as a function of frond numbers over a two week period. Each of the six phytotoxic compounds that we tested significantly reduced *Lemna* growth at every concentration. Final frond counts were all less than 10% of the control. Growth was immediately arrested at all concentrations of PPT, at 50 and 100 ug/mL gentamicin, and at 10mM D-alanine.

	12/17	12/20	12/24	12/27	12/31
Control 1	0	0	0	0	1
Control 2	0	0	0	0	2
Kan 5 ug/mL	0	1	4	5	5
Kan 50 ug/mL	0	1	3	4	5
Kan 100 ug/mL	0	2	6	6	6
Spec 10 ug/mL	0	3	7	8	10
Spec 100 ug/mL	0	3	5	5	6
Spec 200 ug/mL	0	3	4	6	6
Gent 5 ug/mL	0	1	3	4	5
Gent 50 ug/mL	0	0	2	2	--
Gent 100 ug/mL	0	0	1	2	3
PPT 2 mg/L	0	1	4	4	4
PPT 4 mg/L	0	2	4	4	4
PPT 10 mg/L	0	2	4	4	4
D-Ala 1 mM	0	0	0	2	6
D-Ala 3 mM	0	0	0	3	6
D-Ala 10 mM	0	0	3	4	4
D-Ser 1 mM	0	0	0	0	0
D-Ser 3 mM	0	0	0	6	12
D-Ser 10 mM	0	0	0	5	9

Appendix Table 2. Number of Chlorotic Fronds. A substantial percentage of fronds developed chlorosis in the presence of all six compounds. 100% chlorosis was achieved after 14 days under PPT and D-alanine pressure.

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