A conditional marker gene allowing both positive and negative selection in plants

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Selectable markers enable transgenic plants or cells to be identified after transformation and can be divided into positive and negative markers, conferring a selective advantage or disadvantage, respectively, on the transformed plant or cell. We present a novel marker–gene, *dao1* encoding D–amino acid oxidase (DAAO, EC 1.4.3.3), that can be used for either positive or negative selection, depending on the substrate. DAAO catalyses the oxidative deamination of a range of D–amino acids[1] and selection is based on differences in the toxicity displayed by different D–amino acids and their metabolites to plants. Thus, D–alanine and D–serine are toxic to plants, but are metabolised by DAAO into non–toxic products, whereas D–isoleucine and D–valine have low toxicity, but are metabolised into the toxic keto acids 3–methyl–2–oxopentanoate and 3–methyl–2–oxobutanoate, respectively. Hence, both positive and negative selection is possible with the same marker gene. The marker has been successfully

established in *Arabidopsis thaliana*, and proven to be versatile, rapidly yielding unambiguous results, and allowing selection immediately after germination.

The ability to metabolise D–amino acids is widespread in prokaryotes and many eukaryotes[2], but current information suggests that D–amino acid metabolism is severely restricted in plants. However, studies of amino acid transporters from plants have shown that several of these proteins may mediate the transport of both L– and D–enantiomers of amino acids, although the latter usually at lower rates[3,4]. These findings imply that plants are capable of absorbing D–amino acids but have a restricted capacity for D–amino acid metabolism. There are several routes of D–amino acid catabolism, one of the most common being oxidative deamination[2]. D–amino acid oxidase is a well–characterized enzyme, and both its crystal structure and its catalytic mechanism have been determined by high–resolution x–ray spectroscopy[5]. It is a flavoenzyme located in the peroxisome, and its recognised function in animals is detoxification of D–amino acids[2]. In addition, it gives yeasts the ability to use D–amino acids for growth[6]. D–Amino acid oxidases from several different species have been characterized and shown to differ slightly in substrate affinities[7], but in general they display broad substrate specificity, oxidatively deaminating all D–amino acids except D–glutamate and D–aspartate[2]. D–amino acid oxidase activity is found in many eucaryotes[2], but there is no report of DAAO activity in plants.

The low capacity for D–amino acid metabolism in plants has major consequences for the way plants respond to D–amino acids. For instance, our studies of growth responses of *A. thaliana* to D–serine and D–alanine show that these compounds may

inhibit growth even at quite low concentrations (**Fig. 1a, b**). On the other hand, some D–amino acids, like D–valine and D–isoleucine, have minor effects on plant growth (**Fig. 1c, d**). We therefore hypothesized that the toxicity of D–serine and D–alanine could be alleviated by the insertion of a gene encoding a D–amino acid metabolising enzyme. To test this hypothesis, we transformed wild type *A. thaliana* with the *dao1* gene from the yeast *Rhodotorula gracilis* under the control of the CaMV 35S promoter. Exposure of this transgenic plant to D–alanine or D–serine showed that it could detoxify both of these D–amino acids (**Fig. 1a**, **b**). On the other hand, D–valine and D–isoleucine, which are not toxic to wild–type plants, have a strong negative influence on the growth of the DAAO expressing plants (**Fig. 1c, d**). The findings that DAAO expression mitigated the toxicity of D–serine and D–alanine, but induced metabolic changes that made D–isoleucine and D–valine toxic suggest that the enzyme could provide a substrate–dependent, dual–function, selectable marker in plants.

To test this idea we germinated T_1 seeds on different selective media. The T–DNA contained both 35S::*dao1* and pNos::*nptII,* allowing D–amino acid and kanamycin selection to be compared in the same lot of seeds. T_1 seeds were sown on medium containing kanamycin (50 μg ml^{-1}), D–alanine (3 mM) or D–serine (3 mM), and the transformation frequencies found on the different selective media were 2.37%, 2.12% and 1.67%, respectively (**Fig. 2a–c**). D–Alanine had no negative effect on the transgenic plants, even at a concentration of 30 mM, but at this concentration, D–serine induced strong growth inhibition (data not shown). We postulated that the lower number of transgenic plants found after selection on 3 mM D–serine may have been due to the compound slightly inhibiting growth of the transgenic plants at this

concentration. Further studies using lower concentrations corroborated this conclusion, and efficient selection using D–serine was achieved on concentrations lower than 1 mM (**Fig. 1a**). Progenies from the transgenic lines selected on D–serine and D–alanine were later confirmed kanamycin resistant, hence ensuring no wild type escapes in selection with either D–serine or D–alanine.

Selection of seedlings on media containing D–alanine or D–serine was very rapid compared to selection on kanamycin. These D–amino acids inhibited growth of wild type plants immediately after the cotyledons of wild type plants had emerged. Therefore, transformants could be distinguished from non–transformed plants directly after germination. The difference between wild type and transgenic plants following D–amino acid selection treatment was unambiguous, with no intermediate phenotypes. In contrast, intermediate phenotypes are common when kanamycin resistance is used as a selection marker (**Fig. 2c**). Furthermore, wild type seedlings were found to be sensitive to sprayed applications of D–serine and D–alanine. One–week–old seedlings were effectively killed when sprayed on three consecutive days with either 50 mM D–serine or D–alanine, although the sensitivity of wild type plants rapidly decreased with age, presumably as the cuticle and leaves became thicker and reduced uptake by the leaves. Transgenic seedlings were resistant to foliar application of D–alanine or D–serine, so selection on soil was possible (**Fig. 2d**). Transgenic plants grown under D–alanine and D–serine selection conditions developed normally. Early development of transgenic plants from line 3:7, 10:7 and 13:4 was compared with that of wild–type plants by cultivation on vertical agar plates. No differences in biomass, number of leaves, root–length or root architecture were detected for the different sets of plants. Furthermore, growth of soil–cultivated wild–type and

transgenic plants (line 10:7) displayed no differences regarding total number of rosette leaves, number of inflorescences and number of siliqua after four weeks of growth. Also, the phenotypes of 17 individual T_1 lines, which were picked for T–DNA segregation, were studied and found indistinguishable from wild type when grown on soil. A problem sometimes encountered following selection on antibiotics is the growth lag displayed by transformants. This phenomenon is explained as an inhibitory effect of the antibiotic on the transgenic plants[8]. However, unlike seedlings picked from antibiotic selection plates, when transgenic seedlings were picked from D–amino acid selection plates and transferred to soil, their growth and development were not hampered, even temporarily. A possible reason for this difference is that the DAAO scavenging of D–amino acids may effectively remove the D–amino acid in the plants. Furthermore, D–alanine and D–serine may merely provide additional growth substrates, since their catabolic products are carbon and nitrogen compounds that are central compounds in plant metabolism. Quantification of *dao1* mRNA from six independent D–alanine and D–serine resistant lines showed a range of different expression levels (**Fig. 2e**). These different expression levels were mirrored in a range of different DAAO activities (**Fig. 2f**). In spite of these differences in mRNA levels and enzyme activities, no phenotypic variation associated with the D–serine and D–alanine treatment was found, suggesting that the DAAO marker is effective over a range of expression levels.

As described above, D–isoleucine and D–valine were found to inhibit growth of the transgenic plants, but not the wild type plants. Therefore, we tested plants containing the construct described above on two sets of media, one containing D–isoleucine and the other containing D–valine at various concentrations, in order to assess whether

DAAO could also be used as a negative marker. Unambiguous negative selection was achieved when seeds were sown on either D–isoleucine or D–valine at concentrations greater than 10 mM (**Fig. 1c, d**). Thirteen individual DAAO expressing lines were tested for their response to D–isoleucine and all of them were effectively killed, whereas wild type plants grew well, with no sign of toxicity. Similar results were obtained for D–valine, although this compound was found to have a moderately negative effect on wild type plants at higher concentrations (**Fig. 1d**).

The keto acid produced in DAAO catabolism of D–isoleucine is the same as that formed when L–isoleucine is metabolised by the endogenous branched chain amino acid transaminase [EC: 2.6.1.42], namely 3–methyl–2–oxopentanoate (Kyoto Encyclopedia of Genes and Genomes, metabolic pathway website, http://www.genome.ad.jp/kegg/metabolism.html) We suggest that the endogenous transaminase may be specific for the L–enantiomer, so the corresponding D–enantiomer is not metabolised in wild type plants, but only in DAAO expressing plants. The negative effects of L–isoleucine (but not of the D–form) observed on wild type plants, supports this speculation. Incubation of cell–free extracts from *dao1* transgenic line 10:7 with D–isoleucine and D–valine resulted in 15 fold and 7 fold increases in production of 3–methyl–2–oxopentanoate and 3–methyl–2–oxobutanoate, respectively, compared to extracts of wild type plants. Further, 3–methyl–2–oxopentanoate and 3–methyl–2–oxobutanoate proved to impair growth of *A. thaliana* (see Methods), corroborating the suggestion that these compounds, or products of their metabolism, are responsible for the negative effects of D–isoleucine and D–valine on the transgenic plants.

The toxicity of some D–amino acids on organisms is not well understood, and has only occasionally been studied in plants[9]. Apart from *A. thaliana* we have also tested other plant species susceptibility to D–serine, including, poplar, tobacco, barley, maize, tomato and spruce. We found all tested species susceptible to D–serine at concentrations similar to those shown toxic for *A. thaliana*. A proposed mechanism for D–serine toxicity in bacteria is via competitive inhibition of β–alanine coupling to pantoic acid, thus inhibiting formation of pantothenic acid[10]. It is possible to alleviate D–serine toxicity in D–serine sensitive strains of *E. coli* by providing pantothenic acid or β–alanine in the media, but D–serine toxicity in *A. thaliana* could not be mitigated using these compounds (see Methods). A second putative cause of D–amino acid toxicity is through competitive binding to tRNA. Knockout studies of D–Tyr–$tRNA^{Tyr}$ deacylase in *E. coli* have shown that the toxicity of D–tyrosine increases with lack of deacylase activity[11], indicating that D–amino acids interfere at the tRNA level. Similar genes to bacterial deacylase have also been identified in *A. thaliana*[11], corroborating the possibility that the mode of toxic action of D–amino acids might be via competitive binding to tRNA.

The natural occurrence of D–amino acids in plants is generally low, with measurable levels of foremost D–alanine, D–serine, D–glutamine and D–asparagine but no detectable levels of D–valine and D–isoleucine[12]. Hence, the amount and nature of substrates that DAAO may engage under natural conditions would not cause negative effects on plants. In agricultural crops the use of antibiotic and herbicide resistance markers is a matter of public concern[13], and few markers are available that are not based on these mechanisms[14]. Thus, there is a demand for new markers for both research and commercial crop production Alternatives are being sought for and

DAAO has the potential to provide one such option. Further, removing the selectable marker once it has served its purpose is sometimes desirable, and use of DAAO in existing techniques[15] may have advantages over other markers in this respect, since both its insertion and loss can be screened, by positive and negative selection, respectively.

Methods

Vector construction and plant transformation. DNA and RNA manipulation were performed by standard techniques[16]. The yeast *Rhodotorula gracilis* was grown in liquid culture containing 30 mM D–alanine to induce the D–amino acid oxidase gene *dao1*. Total RNA was isolated from the yeast and used for cDNA synthesis. The PCR primers 5′–ATTAGATCTTACTACTCGAAGGACGCCATG and 5′–ATTAGATCTACAGCCACAATTCCCGCCCTA were used to amplify the *dao1* gene from the cDNA template by PCR. The PCR fragments were sub–cloned into the pGEM®–T Easy vector (Promega) and subsequently ligated into the *Bam*HI site of the CaMV 35S expression cassette of the binary vector pPCV702kana[17] giving pPCV702:dao1. The vectors were subjected to restriction analysis and sequencing to check that they contained the correct constructs. *A. thaliana* ecotype Col–0 plants were then transformed by the floral dip method[18] with *Agrobacterium tumefaciens* strain GV3101::pMP110 RK carrying the vector described above. Transgenic T_1 plants were selected on medium containing kanamycin (50 μg ml^{-1}). Lines containing a single T–DNA insertion locus were selected by statistical analysis of T–DNA segregation in the T_2 population that germinated on kanamycin–containing medium. Plants with a single locus of inserted T–DNA were grown and self–fertilized. Homozygous T_3 seed stocks were then identified by analysing T–DNA segregation in

T_3 progenies and confirmed to be expressing the introduced gene by northern blot analyses.

Selection analysis. T_1 seeds were surface–sterilized and sown in Petri plates that were sealed with gas–permeable tape. The growth medium was half strength MS[19] with 0.5% wt/vol sucrose and 0.8% wt/vol agar, plus 3 mM D–alanine, 3 mM D–serine or 50 μg ml^{-1} kanamycin as the selective agent. Plants were grown for five days following germination with a 16 h photoperiod at 24 °C. To evaluate the selection efficiency on different substrates, 2,074, 1,914 and 1,810 T_1 seeds were sown on D–alanine, D–serine and kanamycin selective plates, respectively, and the number of surviving seedlings was counted (44, 32 and 43, respectively).

Toxicity studies. To evaluate the toxic action of 3–methyl–2–oxopentanoate and 3–methyl–2–oxobutanoate, wild type plants were sown on two sets of half strength MS agar plates, each containing one of the compounds in a range of concentrations (0.01–10 mM). Plants were slightly affected by 3–methyl–2–oxopentanoate at 0.1 mM, and total growth inhibition was observed at 1 mM. For 3–methyl–2–oxobutanoate, 5 mM was required for complete inhibition (data not shown). Further, several attempts were made to probe the nature of D–serine's toxicity. In accordance with studies on *E. coli*[10], we tried to rescue wild type *A. thaliana* grown on lethal concentrations of D–serine through amendments with five potential inhibitors of D–serine toxicity (L–serine, Ca–pantothenate, β–alanine, leucine and threonine) added both separately and in combinations in a very wide range of concentrations (0.001–50 μg ml^{-1}), without any success.

Enzyme assays. Soluble proteins were extracted by extracting 0.1 g samples of plant material that had been finely pulverized in a 1.5 ml eppendorf tube in 1 ml of 0.1 M potassium phosphate buffer, pH 8. D– amino acid oxidase activity was then assayed as follows. Reaction mixtures were prepared containing 2,120 μl of 0.1 M potassium phosphate buffer (pH 8), 80 μl of crude protein extract and 100 μl of 0.3 M D–alanine. The samples were incubated for 2 hours at 30° C. The enzyme activity was then assessed, by measuring the increase in absorbance at 220 nm (ΔE = 1,090 M^{-1} cm^{-1}) associated with the conversion of D–alanine to pyruvate, after transferring the test tubes to boiling water for 10 min to stop the reaction. In control reactions D–alanine was added immediately before boiling. One unit of DAAO activity is defined as the turnover of one micromole of substrate per min, and activity was expressed per gram plant biomass (fresh weight). The breakdown of D–isoleucine and D–valine in DAAO incubations, and the associated production of 3–methyl–2–oxopentanoate and 3–methyl–2–oxobutanoate, were analysed by HPLC[20]. In other respects the reactions were performed as described above.

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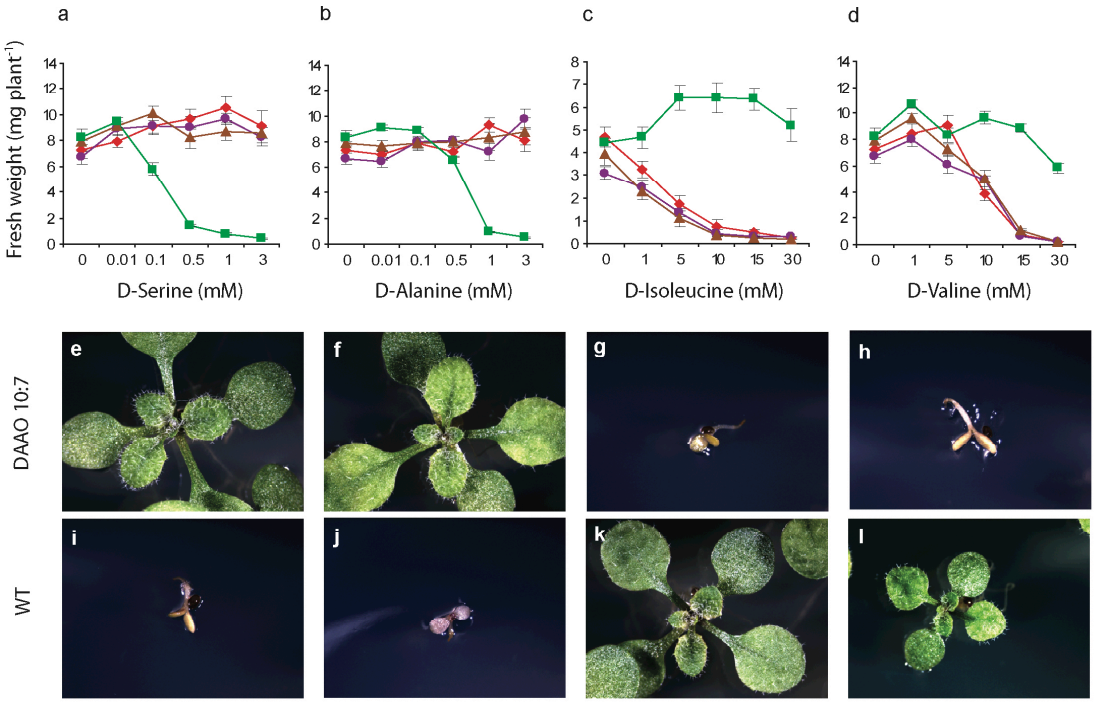
Figures

Figure 1. D–amino acid dose–response curves of *dao1* transgenic and wild type *Arabidopsis*. (**a**–**d**) Growth of *dao1* transgenic line 3:7 (◆), 10:7 (◆), 13:4 (▲) and wild type (■) plants, in fresh weight per plant, on media containing various concentrations of D–serine, D–alanine, D–isoleucine and D–valine in half strength MS with 0.5% wt/vol sucrose and 0.8% wt/vol agar. Please note the different concentration ranges used for the different D–amino acids. The plants were grown for 10 days following germination under 16 h photoperiods at 24 °C; n=10 ± SE, except for plants grown on D–isoleucine, where smaller Petri dishes were used, (n=6 ± SE), (**e**–**h**) and (**i**–**l**) Photographs of *dao1* transgenic line 10:7 and wild type plants, respectively, grown for 10 days on the highest concentrations of the D–amino acids shown in the respective graphs above. All pictures have the same magnification.

Figure 2. Selection of primary transformants with the DAAO marker. DAAO T_1 seedlings on media containing (**a**) 3 mM D–alanine, (**b**) 3 mM D–serine and (**c**) 50 μg ml^{-1} kanamycin. Seeds were surface sterilised and sown on half strength MS plates with 0.5% wt/vol sucrose, 0.8% wt/vol agar and the respective selective compound, then grown for five days following germination under 16 h photoperiods at 24 °C. (**d**) DAAO transgenic plants grown on soil photographed following selection by spraying with (i) D–alanine and (ii) D–serine, and wild type plants sprayed with (iii) D–alanine and (iv) D–serine. Eight seeds were sown per plot and treatment, on soil, and grown for seven days following germination before applying the selective treatment, which consisted of spraying with aqueous 50 mM solutions of D–alanine or D–serine with 0.05% Tween 80 on three consecutive days. (**e**) Northern blot analysis of *dao1* mRNA

levels from six D–serine and D–alanine resistant lines and wild type plants. Ten μg total RNA was loaded per lane and separated on an agarose gel. Ethidium bromide–stained total RNA bands are shown as loading controls. (**f**) D–Amino acid oxidase activity in six transgenic lines and wild type. A unit of DAAO activity is defined as the turnover of one micromole of substrate per min. Bars represent means, n=3 ± SE.


a
b
c
d
Fresh weight (mg plant-1)
D-Serine (mM)
D-Alanine (mM)
D-Isoleucine (mM)
D-Valine (mM)
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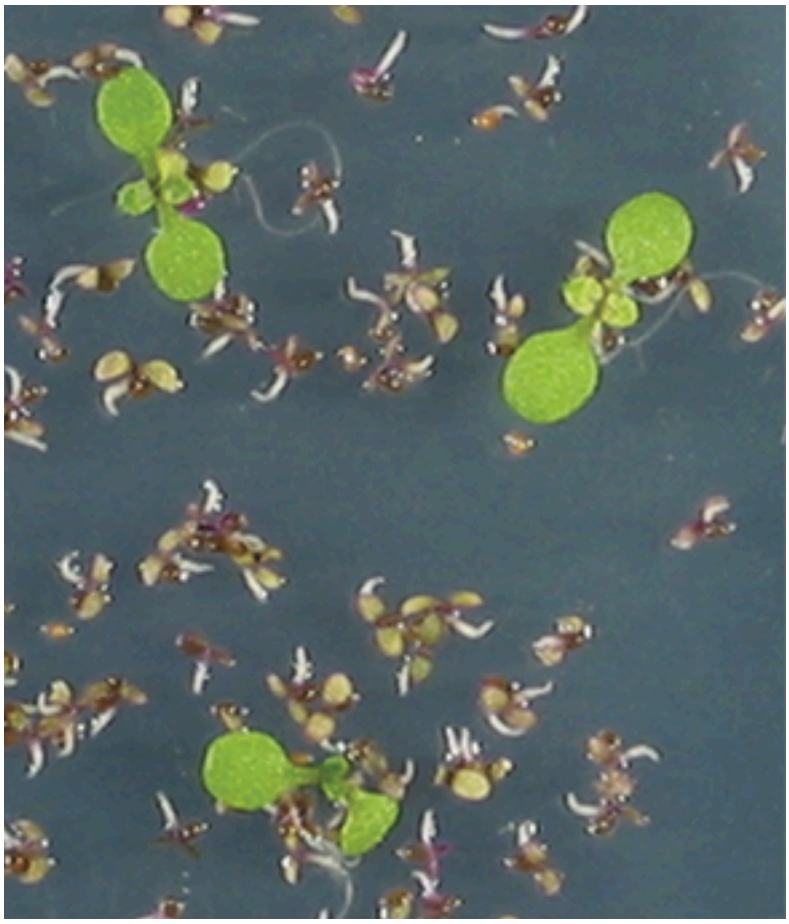

Figure 2a

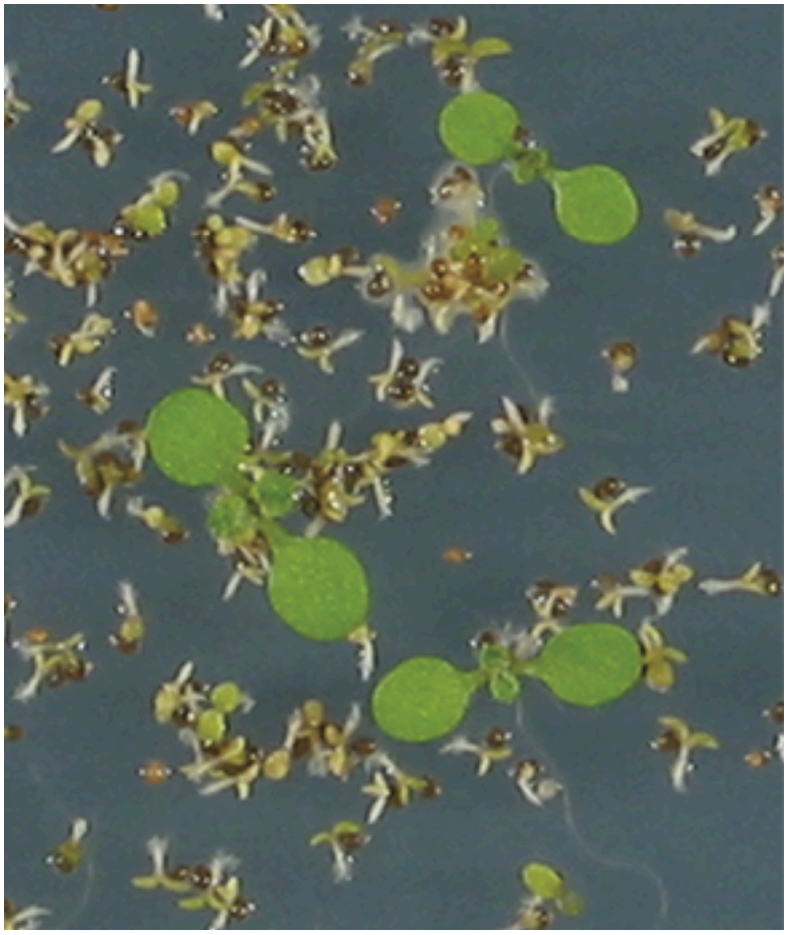

Figure 2b

Figure 2c

Figure 2d

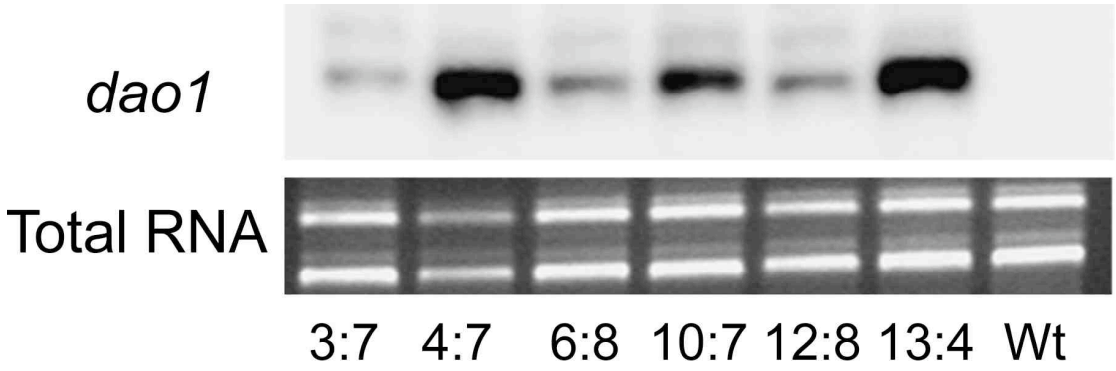

Figure 2e

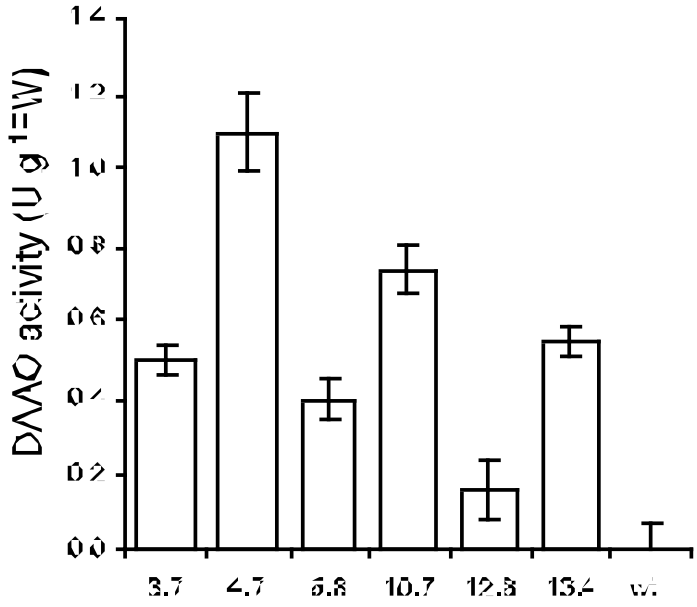

Figure 2f