D-Lactate dehydrogenase as a marker gene allows positive selection of transgenic plants

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ABSTRACT

D-Lactate negatively affects *Arabidopsis thaliana* seedling development in a concentration-dependent manner. At media D-lactate concentrations greater than 5–10 mM the development of wild-type plants is arrested shortly after germination whereas plants overexpressing the endogenous D-lactate dehydrogenase (D-LDH) detoxify D-lactate to pyruvate and survive. When the transgenic plants are further transferred to normal growth conditions they develop indistinguishably from the wild type. Thus, D-LDH was successfully established as a new marker in *A. thaliana* allowing selecting transgenic plants shortly after germination. The selection on D-lactate containing media adds a new optional marker system, which is especially useful if the simultaneous selection of multiple constructs is desired.

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1. Introduction

After transformation, transgenic plants are identified by means of selection markers. In traditional plant selection systems, selectable marker genes encode herbicide tolerance (e.g., paraquat, BASTA, etc.) or resistance to antibiotics (e.g., kanamycin, ampicillin, hygromycin, etc.), most of which may pose potential danger to human health or the environment [1]. Here, we present a novel, alternative selection marker system that is based on the metabolism of D-lactate. D-Lactate results from the glyoxalase system, which catabolizes methylglyoxal (MG), a compound formed as a byproduct of glycolysis in all type of cells through non-enzymatic phosphate elimination from triose phosphates (dihydroxyacetone phosphate and glyceraldehyde 3-phosphate; [2]) (Fig. 1). The glyoxalase system comprises (i) glyoxalase I, an enzyme that catalyzes the formation of S-D-lactoylglutathione from the hemithioacetal formed non-enzymatically from MG and glutathione an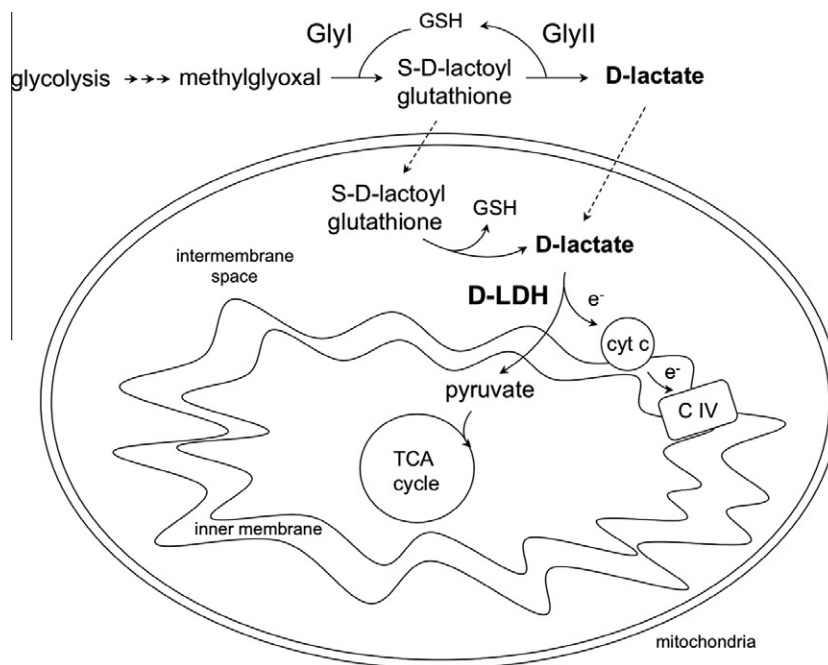d (ii) glyoxalase II, which catalyzes the hydrolysis of S-D-lactoylglutathione to regenerate glutathione and liberate D-lactate [3,4]. D-Lactate is further converted to pyruvate by D-lactate dehydrogenase (D-LDH) (Fig. 1). The *Arabidopsis thaliana* D-LDH is encoded by a single gene (At5g06580), localizes to mitochondria, and uses cytochrome c as electron acceptor [5]. Moreover, this enzyme shows a high specificity for D-lactate with a catalytic efficiency 200- and 2000-fold higher than that for L-lactate and glycolate, respectively [5].

In this work, we present the generation of plants that constitutively overexpress *A. thaliana* D-LDH using the Cauliflower Mosaic Virus 35S (CaMV35S) promoter. D-LDH-overexpressing plants are able to metabolize and detoxify externally provided D-lactate and MG, while wild-type plants accumulate toxic levels of these metabolites. Consequently, transformants are able to grow and develop in the presence of increased of D-lactate in the media whereas the development of wild-type plants is arrested shortly after germination. These features were used to develop D-LDH as a marker that allows mutant selection in the next generation of transformed plants.

2. Materials and methods

2.1. Cloning of pGWB2-D-LDH expression vector

Total RNA was extracted from 100 mg leaf material from 4-week-old *A. thaliana* plants using the TRIzol reagent (Invitrogen). One microgram of RNA was reverse-transcribed into cDNA by the

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Fig. 1. Metabolic pathway of D-lactate production and catabolism. D-Lactate results from methylglyoxal through the action of glyoxylase I (GlyI) and glyoxylase II (GlyII). D-Lactate is converted to pyruvate through mitochondrial D-LDH while electrons are transferred to the respiratory chain through cytochrome c (cyt c) in the intermembrane space (Engqvist et al., 2009). Dashed arrows represent possible transport processes. CIV, complex IV. e⁻, electron. GSH, glutathione. TCA cycle, tricarboxylic acid cycle.

SuperScriptII reverse transcriptase (Invitrogen) and the D-LDH coding sequence was PCR-amplified using primers AtD-LDH-FW1 (5′-caccATGGCTTTCGCTTCAAAATTC-3′) and AtD-LDH-RV1 (5′-GA AACATACATGAGGAGGAATTAACTTTC-3′) in a reaction with PfuTurbo DNA polymerase (Stratagene). The resulting PCR product was then directionally cloned into the pENTR/D-Topo vector (Invitrogen) and the resulting pENTR/D-Topo-D-LDH vector was sequenced using the PRISM fluorescent dye-terminator system (Applied Biosystems). To generate plant expression vectors, the attL-sites in pENTR/D-Topo-D-LDH were recombined with the attR-sites in pGWB2 [6] using LR recombinase (Invitrogen). pGWB2 contained the CaMV35S promoter. The correct identity of the resulting pGWB2-D-LDH construct was controlled by digestion of isolated vector DNA using several sets of endonucleases followed by agarose-gel analysis. All cloning steps were performed in the *E. coli* strain DH5α.

2.2. Plant transformation and selection

A. thaliana wild-type (Columbia-0) and the overexpression lines were grown in pots containing three parts of soil (Gebr. Patzer KG, Sinntal-Jossa) and one part of vermiculite (Basalt Feuerfest, Linz) in a growth cabinet in a 16/8 h light/dark cycle at 22 °C day/18 °C night temperatures and at a photosynthetically active photon flux density of 100 µmol quanta m^{-2} s^{-1}. The pGWB2-D-LDH construct was transformed into *Agrobacterium tumefaciens* strain GV3101 and subsequently introduced into *A. thaliana* wild-type plants by vacuum infiltration [7]. To evaluate the selection efficiency on different compounds, the resulting pGWB2-D-LDH transformants (35S::D-LDH plants) were selected on sterile MS agar plates with kanamycin (50 mg/L) or D-lactate (10 mM) and the number of surviving seedlings was counted. The presence of the correct overexpression construct in the transformants was confirmed by PCR analysis on DNA extracted from leaf material. The transgenic plants were subsequently allowed to self-pollinate until non-segregating T3 lines were obtained. All further analyses were performed with homozygous plants.

2.3. Extraction of RNA and RT-PCR analysis

To analyse the expression of the transgene, total RNA from leaves was isolated from 100 mg of tissue using the TRIzol reagent (Gibco-BRL). RNA was converted into first strand cDNA using the SuperScriptII Reverse Transcriptase (Invitrogen). PCR reactions were conducted in a final volume of 10 µl using 0.5 µl of the transcribed product and Taq DNA polymerase (Qiagen). The primers used were AtD-LDH-FW2 (5′-tgtccctttatctcaccttgc-3′) and AtD-LDH-RV2 (5′-tc tgctcttcactgcttgga-3′) As control, the *actin2* gene was amplified using the primers Actin-FW (5′-ATGGAAGCTGCTGGAATCCAC-3′) and Actin-RV (5′-TTGCTCATACGGTCAGCGATG-3′). PCR products were resolved on a 1.0 % (w/v) agarose gel.

2.4. Studies of toxicity

The toxicity of MG and D-lactate was assayed in a range of concentrations (0.1–5 mM MG and 0.5–20 mM D-lactate) on agar plates containing MS medium. For root length experiments, seedlings were grown on vertical MS agar plates containing a range of D-lactate concentrations and roots were measured against a ruler at 4, 8 and 12 days after imbibition.

2.5. Statistical analysis

Significance was determined according to the two-sided Student's *t*-test using the Excel computer program (Microsoft Corp.).

3. Results and discussion

We showed previously that (i) D-lactate and MG negatively affect *A. thaliana* seedling development in a concentration-dependent manner and that (ii) *d-ldh* mutants i.e., plants lacking an active D-LDH, show lower tolerance than wild-type plants, indicating an impaired detoxification of these substances caused by the loss of D-LDH activity [5]. We therefore hypothesized that D-LDH overexpressing plants would be more resistant to these substances. To

test this, we produced *A. thaliana* plants constitutively overexpressing D-LDH (*35S::D-LDH* plants). Seeds from wild-type as well as from *35S::D-LDH* plants were germinated on MS plates in the presence of either MG (0.1–1 mM) or D-lactate (5–20 mM). In accordance with our hypothesis, the constitutive overexpression of D-LDH mitigated the negative effects on growth of MG up to 0.1 mM and of D-lactate up to 10 mM (Fig. 2). *35S::D-LDH* overexpressing lines grew better than the wild type in the presence of MG up to 0.5 mM. At higher MG concentrations similar toxic effects were found in all genotypes, probably because glyoxylase I and/or glyoxylase II may control of

the flux through the pathway. On the other hand, *35S::D-LDH* overexpressing lines grew better than the wild type in the presence of D-lactate in all tested concentration (Fig. 2). It is worth mentioning that at all D-lactate concentrations tested *35S::D-LDH* plants showed cotyledons and true leaves at the same developmental stage as plants grown on media without D-lactate (Fig. 2). To illustrate the better performance of *35S::D-LDH* plants over wild type in the presence of D-lactate, the effect of increasing concentrations of this compound on root elongation was analyzed at 4, 8 and 12 days after imbibition in two independent *35S::D-LDH* lines. Fig. 3 shows a
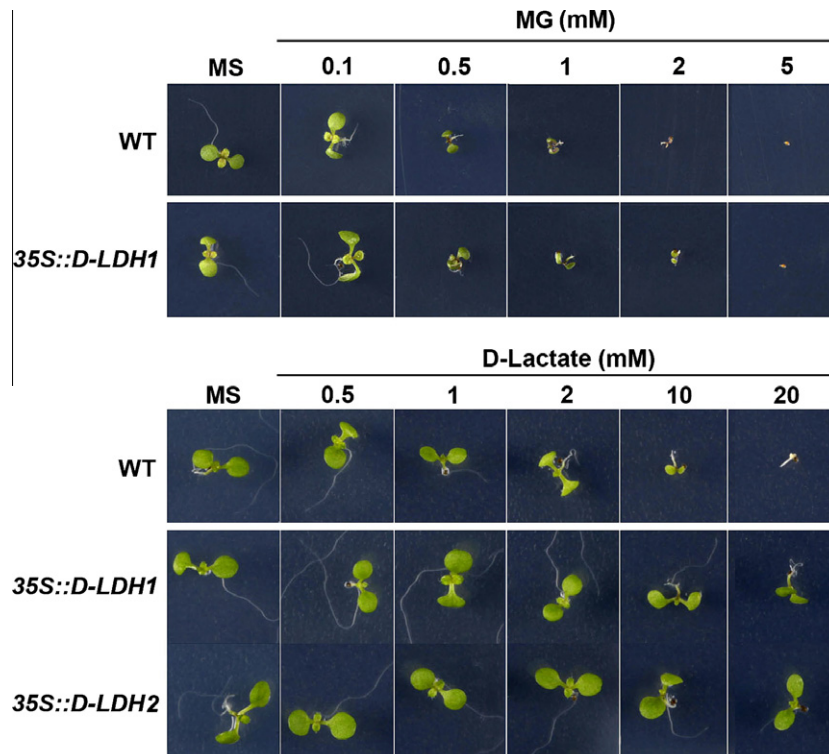
Fig. 2. Effect of methylglyoxal (MG) and D-lactate on the growth of *A. thaliana* seedlings. Wild-type plants and two independent homozygous D-LDH overexpressing lines derived from the same construct were grown for 8 days on MS solid media and on the same media supplemented with different concentrations of MG (0.1–5 mM) or D-lactate (0.5–10 mM).
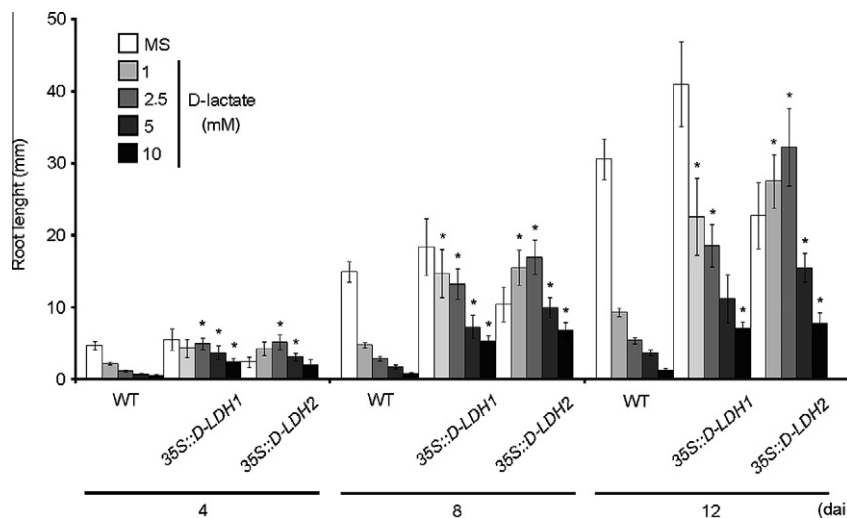

Fig. 3. D-Lactate dose responses of wild-type and D-LDH overexpressing plants. Root-length of wild-type and *35S::D-LDH1* and *35S::D-LDH2* plants grown on MS media containing different concentrations of D-lactate measured at 4, 8 and 12 days after seed imbibition (dai). Error bars indicate standard errors of measurements. Asterisks indicate significant differences to wild-type values at each time point calculated by a two-sided Student's *t*-test ($P < 0.05$).

dose-dependent reduction of root growth in wild type and the D-LDH overexpressing lines. While the wild type showed a continuous reduction in root length with increasing concentrations of D-lactate, 35S::D-LDH lines showed this dose-dependent toxic effect only at very high concentrations (5–10 mM D-lactate). Moreover, the developmental differences between the wild type and the overexpressing lines could unambiguously be observed already at 8 days after imbibition (Figs. 2–4). Growth on 5–10 mM D-lactate seemed ideal to most clearly distinguish transformed from untransformed plants. At this early developmental stage as the wild-type seedlings remained in the early cotyledonary stage and were yellowish while the 35S::D-LDH developed normal true leaves and roots. If plants were allowed to further grow in this condition, untransformed (wild-type) plants also developed the first true leaves. However, the root elongation was completely blocked (Fig. 4, 12 dai) and these plants died within the following week

Fig. 4. Selection of T1 transformants. Comparative growth of 35S::D-LDH T1 seedlings on MS media containing either D-lactate (10 mM) or kanamycin (50 mg/l) at 8 and 12 days after imbibition (dai).

(Fig. 5, 16 dai). If even shorter selection times are desired higher D-lactate concentrations in the media could be used. For example, at 20 mM D-lactate untransformed (wild-type) plants did not develop further (Fig. 2). However, since primary root elongation of 35S::D-LDH was slightly affected by growth on D-lactate concentrations higher than 20 mM (Fig. 2) further selection was performed with 10 mM D-lactate.

The binary vector used to produce 35S::D-LDH lines contained both CaMV35S::D-LDH and pNos::nptI, thereby allowing the comparative selection of a similar amount of T1 seeds on D-lactate or kanamycin (Fig. 4). RT-PCR analyses confirmed that the selected plants indeed harbored and expressed the transgenes (not shown). Using 10 mM D-lactate or 50 mg/l kanamycin, the transformation frequencies obtained 8 days after imbibition were 1% (total seeds plated: 1900; resistant individuals: 20) and 1.7% (total seeds plated: 1953; resistant individuals: 33), respectively. Furthermore, the difference in growth rate between transgenic and non-transgenic plants on plates containing D-lactate is sufficiently large to easily detect transformants by visual inspection (Fig. 4). This demonstrates that the selection of transformants grown on D-lactate, although resulting in a lower number of individuals compared to kanamycin, was highly stringent and as rapid as the selection on kanamycin. Moreover, when these plants were transferred to greenhouse conditions they showed no obvious phenotypic differences compared to wild-type plants (not shown), indicating that D-LDH overexpression did not affect further plant development. Finally, the effect of D-lactate and its physiological precursor MG on the growth of other species like tomato and tobacco was tested. As shown in Fig. 5, both compounds are also toxic for these species, indicating the potential use of D-lactate as a selection marker for plants of agricultural interest. Some conditional-positive selection systems are more effective in certain species and regeneration systems than others, e.g., kanamycin has lower selection efficiency in cereals than in dicots. However, very small differences in fitness that may be trivial under laboratory conditions can have significant
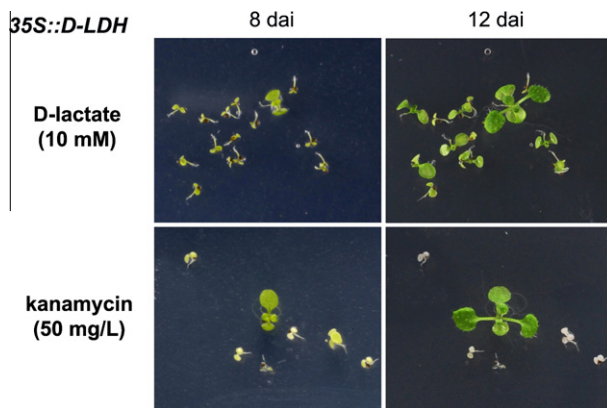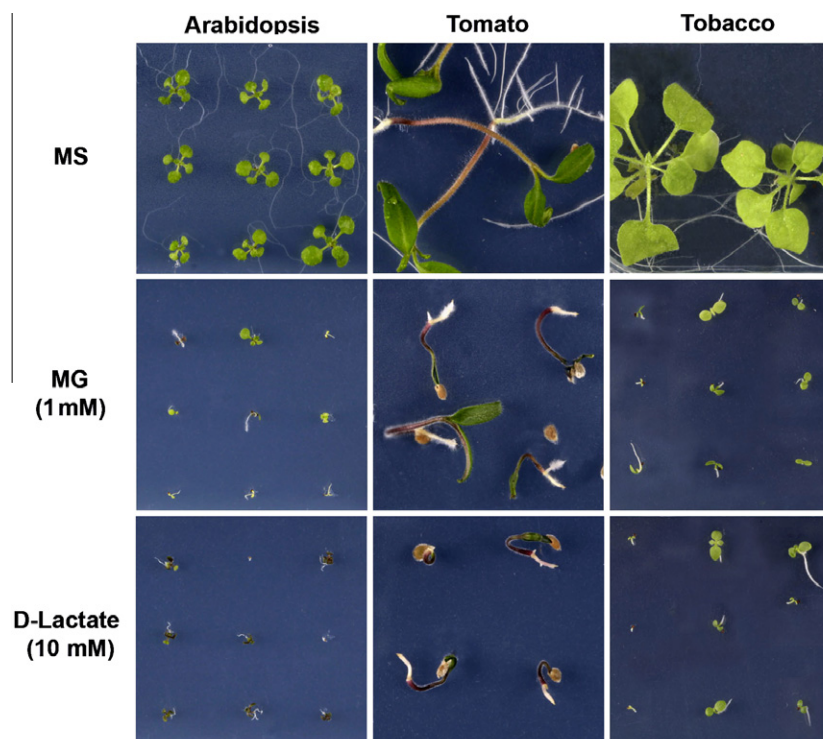
Fig. 5. Effect of methylglyoxal (MG) and D-lactate on plant growth. Wild-type A. thaliana, tomato and tobacco seedlings either grown on MS or MS containing either 1 mM MG or 10 mM D-lactate at 16 days after imbibition (dai).

effects on the total yield of a crop under field conditions. Thus, it remains to be tested whether overexpressing D-LDH might have any effects on crop yield.

4. Concluding remarks

There certainly is a need for novel selectable markers to minimize the use of antibiotics, herbicides and resistance-causing genes in plant sciences. There is also currently a strong public opinion against using antibiotic markers for producing transgenic crop plants. The selection on D-lactate adds a new marker system to molecular plant- and breeding science providing a good alternative to the traditional markers (especially antibiotics). Furthermore, this new marker extends the range of available alternative selection systems, which is important when multiple constructs should be selected simultaneously in the context of stepwise transformations.

The overexpression of D-LDH may not cause negative effects on the transgenic plants because D-LDH participates in an endogenous detoxification pathway and has high substrate specificity [5]. However, one could argue that the constitutively expressed D-LDH might interfere with plant metabolism during further growth and/or lead to a potential deregulation of other genes. It should also be considered that overexpression of an endogenous gene as a selectable marker could lead to co-suppression of the transgene and/or the endogenous gene in subsequent generations [8–10], leading to a negative impact on the yield of transgenic crops lines. To avoid these consequences, D-LDH could be expressed under the control of an inducible promoter or a promoter that would only confer expression during early developmental stages. We expect that this alternative selection system will become a useful new tool in plant sciences, also addressing public concerns against antibiotic-based selection markers at the same time.

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