Use of basta selection for genetic transformation of Regen SY alfalfa

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Introduction

Genetic transformation is a powerful tool to understand developmental, biochemical, and physiological processes in plants, as well as to create plants with novel traits that might be difficult or impossible to achieve through conventional breeding. Genotypes from Regen SY alfalfa germplasm are amenable to Agrobacterium-mediated transformation and regeneration using a well-established protocol [1,2]. In this protocol, a marker gene, often kanamycin or hygromycin resistance, allows selection of transformed cells using the corresponding antibiotic. One approach to understanding multigene phenomena is to supertransform previously transformed plants using multiple selectable markers. We sought to use basta (phosphinothricin, PPT) resistance (conferred by the bar gene) as a selectable marker to supertransform plants that were already resistant to kanamycin and hygromycin.

Pathway engineering

We previously showed [3] that a biosynthetic pathway for phaselic acid (caffeoyl-malate) could be engineered into alfalfa by expression of red clover HMT and downregulation of endogenous CCOMT (Fig. 1).

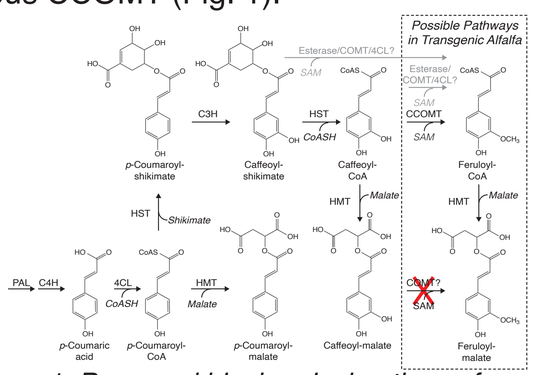

Figure 1. Proposed biochemical pathway for synthesis of phaselic acid in alfalfa.

Because the engineered alfalfa accumulated substantial levels of *p*-coumaroyl-malate in addition to phaselic acid, we hypothesized that overexpression of HST and C3H by supertransformation might result in increased levels of phaselic acid. The engineered alfalfa were generated with kanamycin and hygromycin selectable markers, so we sought to use basta selection.

Transformation Constructs

A "stacked" gene construct to express HST and C3H and a control construct expressing GFP were made in pYB3301 [4], which has the Bar selectable marker.

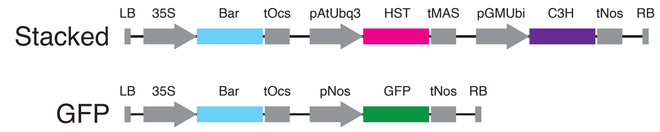

Figure 2. Schematic of gene construct used for transformation. Only the T-DNA portion is shown.

Basta selection

The mode of action of PPT is inhibition of glutamine synthetase (Fig. 3), leading to reduced glutamine and ammonia accumulation.

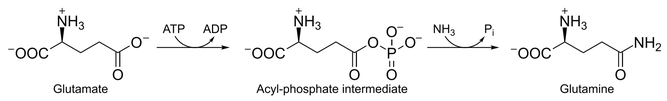

Figure 3. Glutamine synthetase reaction.

Table 1. Media for alfalfa transformation as described by Austin et al. [1].

Medium	Purpose
B5h	Co-cultivation, callus induction, pro-embryo formation
B5h0	Embryo development
MMS	Shoot development, rooting

Preliminary transformation experiments using the method of Austin et al. [1,2], required high amounts of PPT (8 mg/L) for selection, presumably because B5h/B5h0 media (Table 1) as originally formulated are supplemented with "Stock Amino Acids" that results in glutamine-rich (800 mg/L) medium. We therefore developed a "kill curve" for PPT with B5h reformulated without glutamine (Figure 4).

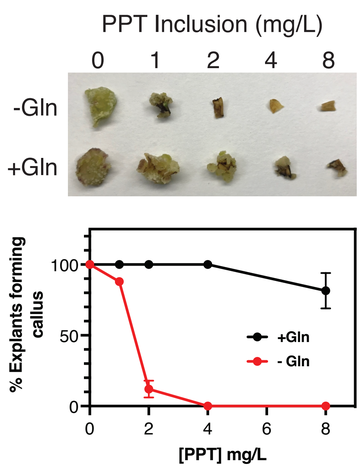

*Figure 4. Callus formation on alfalfa leaf explants after 2 weeks on B5h medium at various levels of PPT without (-) or with (+) glutamine (Gln). (*Top*) Typical leaf explants. (*Bottom*) Percent explants forming callus +/- SEM. Average of 2 experiments with at least 9 explants per treatment per experiment.*

Results

We carried out transformation of Regen SY27 alfalfa using the protocol of Austin et al. [1,2], except we eliminated supplemental glutamine from B5/B5h0 medium. PPT was used for selection at 2 mg/L for B5h, 2 mg/L for B5h0, and 0-1 mg/L for MMS. No embryos or shoots were recovered from alfalfa explants not cocultivated with agrobacterium. So far, we have analyzed 4 independent events for the stacked construct, and 7 independent events for the GFP construct by PCR for the presence of the bar selectable marker (Fig. 5). The bar gene was only detected in 2 of the GFP plants. We have at least 4 additional independent events for each construct (rooted shoots) still to be analyzed.

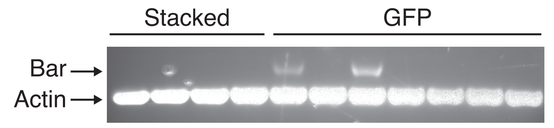

Figure 5. Presence of the bar (basta resistance) gene in several transgenic alfalfa independent events was assessed by PCR. Amplification of endogenous actin genes served as a positive control.

Conclusions

- Glutamine is not required for regeneration of Regen SY alfalfa.
- Recovery of alfalfa with the bar transgene indicates that transformation was successful.
- High recovery of escapes (plants without the bar transgene) suggests further optimization of selection is needed.
- Plants having the bar transgene will be further analyzed for the presence of HST/C3H or GFP.

References & Acknowledgements

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