Successful protoplast transfection in *Cichorium* and the search for bitterness related genes

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Introduction

Cichorium intybus var. *sativum* (chicory) and var. *foliosum* (witloof) are economically important crops in Belgium with a high nutritional value. Many specialized metabolites have been isolated, such as polyphenols and terpenoids, with beneficial effects on human health. *Cichorium* is rich in sesquiterpene lactones (SL) responsible for the bitter taste. CRISPR/Cas9 genome editing of *Cichorium* species could alter specific SL genes which could lead to altered bitterness.

CRISPR/Cas9 transfection and regeneration of plants

In this study the new breeding technique CRISPR/Cas9 was used on witloof 'Van Hamme', using a protoplast transfection method (Fig. 1). A transfection efficiency of 24,04±5,88% was obtained. A CRISPR/Cas9 vector (pCAS9_PDS) was designed to target the *phytoene desaturase gene* (*CiPDS*), which leads to a dwarf albino plant phenotype (Fig. 1). Regeneration of transfected protoplast into plants showed a mutation efficiency of 20.66±2.42%. Mostly frameshift mutations occurred in the albino plants leading to a knock-out of the *PDS* gene.

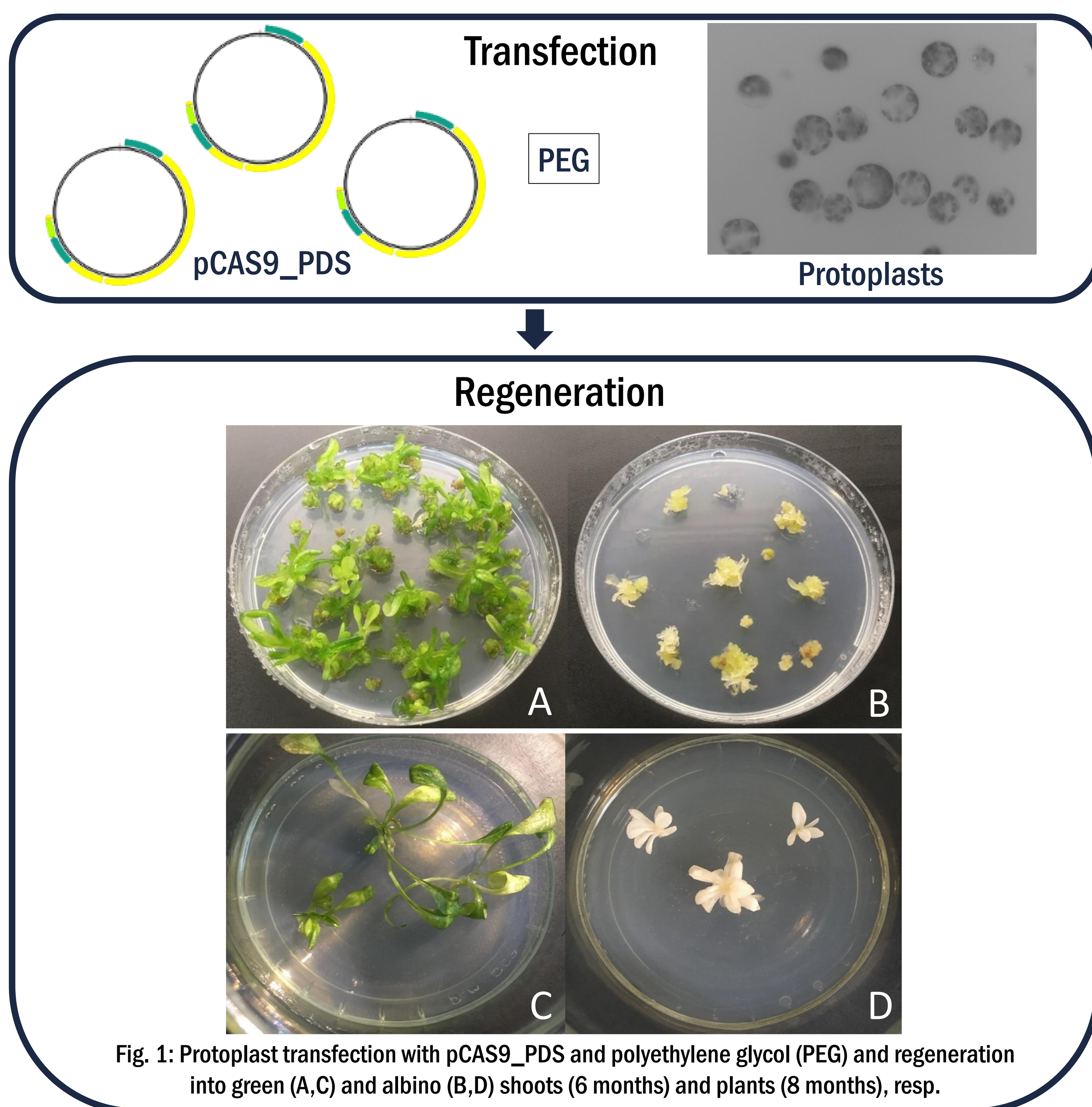

Fig. 1: Protoplast transfection with pCAS9_PDS and polyethylene glycol (PEG) and regeneration into green (A,C) and albino (B,D) shoots (6 months) and plants (8 months), resp.

This successful protoplast transfection and regeneration protocol can now be used to target genes of the SL pathway. Three genes have already been identified to control the production of SLs*: germacrene A synthase (GAS), germacrene A oxidase (GAO)* and *costunolide synthase (COS).* CRISPR/Cas9 vectors containing sgRNA targets for *GAS*, *GAO* or *COS* were used for protoplast transfection and plant regeneration. These plants are currently screened for mutation events.

Discovering new genes

To discover new genes related to the SL pathway, a set of samples was generated from three different species: chicory, witloof and lettuce. Two week old seedlings were elicited with the phytohormone jasmonate (MeJA), a known activator of SL biosynthesis, and harvested at three different time points (2h, 6h and 24h). Using *GAS*, *GAO* and *COS* as target genes, RT-qPCR experiments showed the highest upregulation in seedlings harvested after 6 hours of MeJA treatment (Fig. 2). This optimal time point in the three different species was selected for RNA-sequencing. Co-expression analysis with the *GAS, GAO* and *COS* genes will be used to identify candidate genes of the SL pathway.

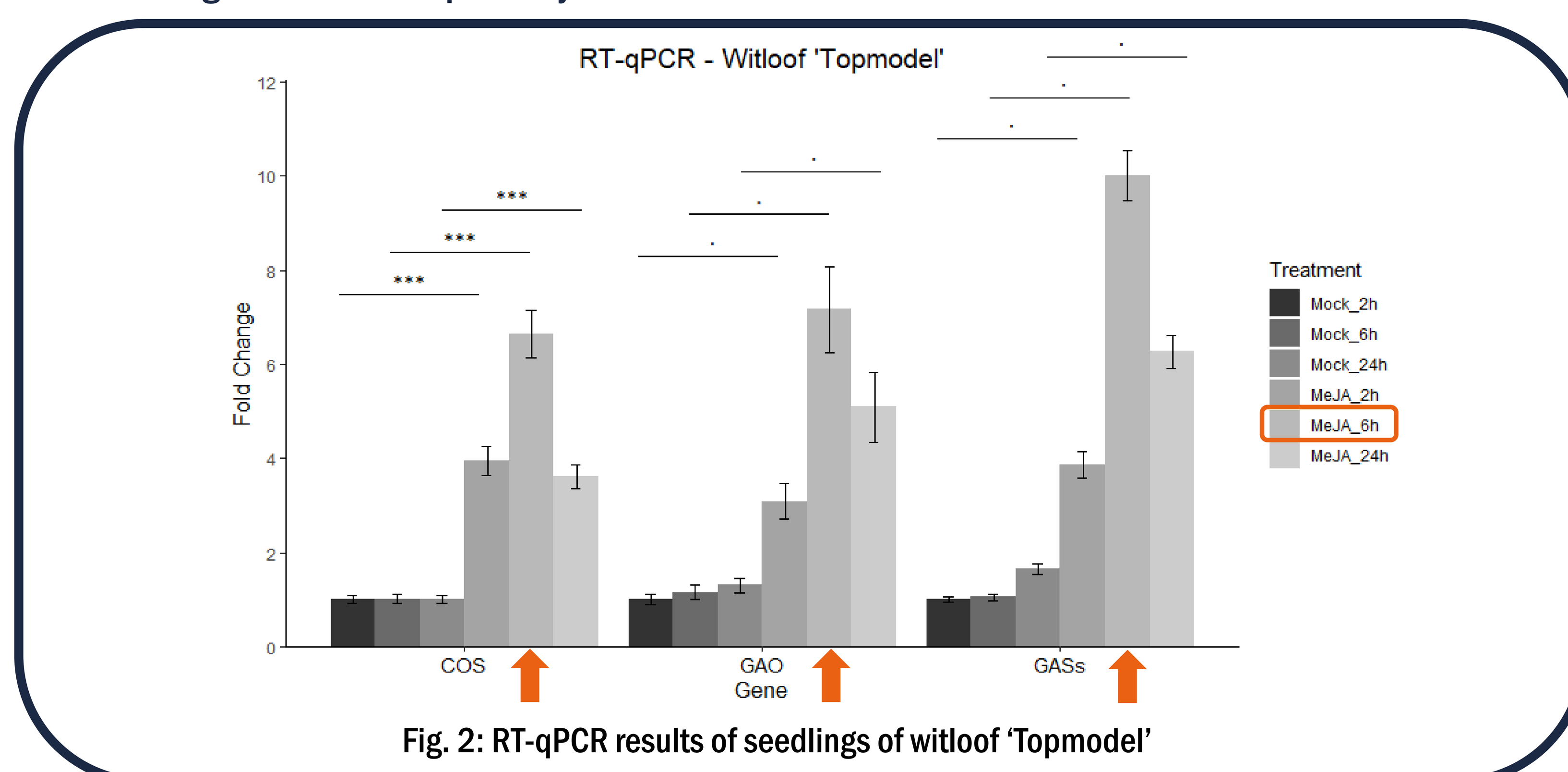

Fig. 2: RT-qPCR results of seedlings of witloof 'Topmodel'

Yeast expression assay

To confirm the functional activity of newly identified candidate enzymes, a functional yeast expression assay will be carried out. The candidate sequences will be cloned into a yeast expression vector and tested by co-transformation with *GAS, GAO* and *COS* genes (Fig. 3). Yeast extracts will be analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) and can confirm enzymatic activity of the encoding genes. A *Nicotiana benthamiana* (tobacco) assay will be executed in parallel.

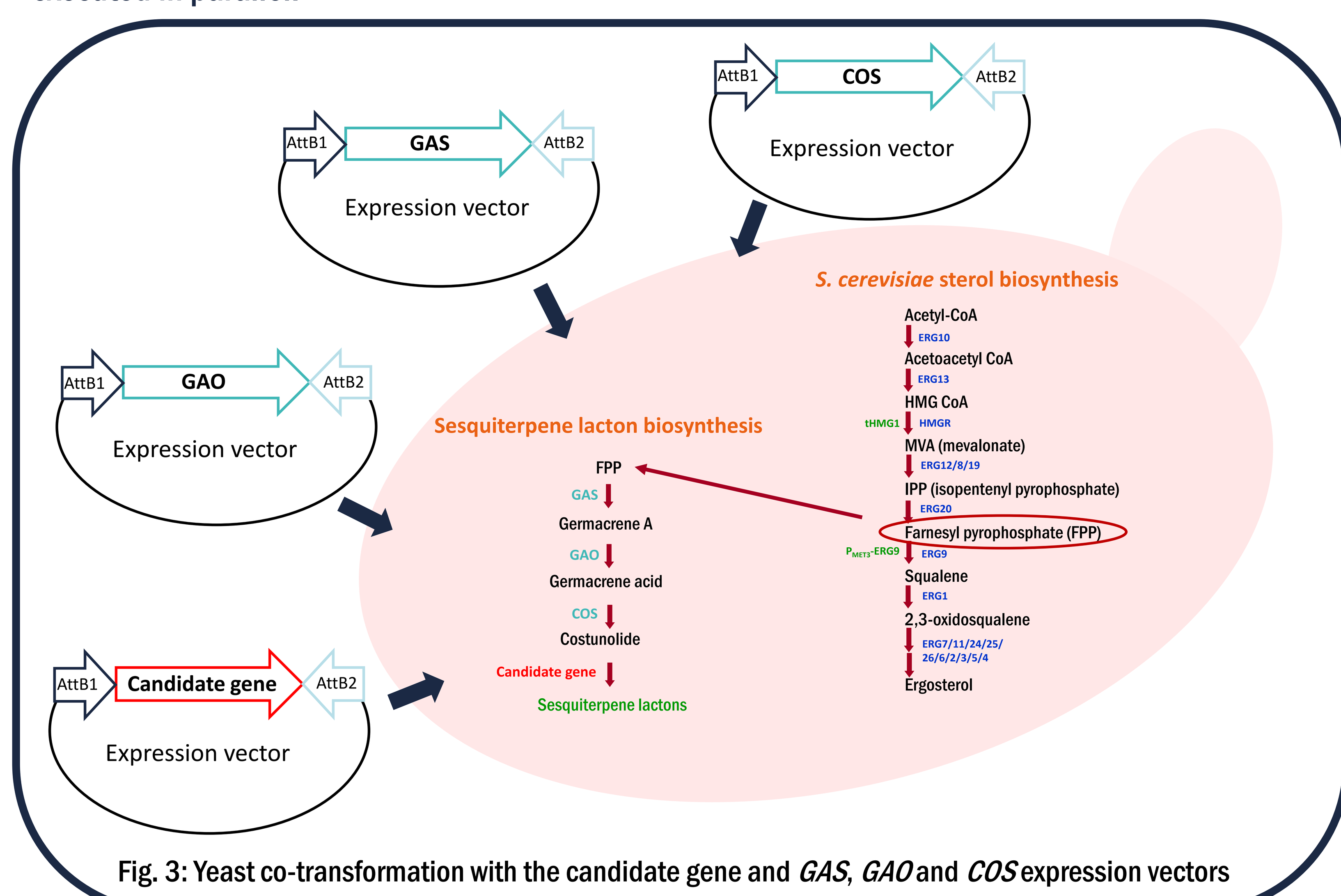

Fig. 3: Yeast co-transformation with the candidate gene and *GAS, GAO* and *COS* expression vectors

Conclusion

A successful protoplast transfection and regeneration method was established to target specific genes in *Cichorium*. To alter the biterness pathway, the three known genes (*GAS, GAO, COS*) have been targeted and are currently beeing screened for mutation events. To discover new genes related to the SL pathway, a differential expression assay has been set-up to generate samples for RNA-seq. This data will be used to execute a co-expression analysis with the *GAS, GAO* and *COS* genes for identification of new candidate SL genes. The functional activity of the newly identified candidate enzymes can be confirmed with the use of a yeast and tobacco expression assay. Identifying these new genes related to the SL pathway and using the successful CRISPR/Cas9 transfection and regeneration protocol opens opportunities to change the bitterness of the *Cichorium* species.

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