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Article Addendum

Gene trap-based identification of a guard cell promoter in Arabidopsis

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Preserving crop yield under drought stress is a major challenge for modern agriculture. To cope with the detrimental effects of water scarcity on crop productivity it is important to develop new plants with a more sustainable use of water and capable of higher performance under stress conditions. Transpiration through stomatal pores accounts for over 90% of water loss in land plants. Recent studies have increased our understanding of the networks that control stomatal activity and have led to practical approaches for enhancing drought tolerance. Genetic engineering of target genes in stomata requires effective expression systems, including suitable promoters, because constitutive promoters (i.e., *CaMV35S*) are not always functional or can have negative effects on plant growth and productivity. Here we describe the identification of the *CYP86A2* guard cell promoter and discuss its potential for gene expression in stomata.

Plants have evolved different strategies to resist drought, of which the best understood is the closure of stomatal pores induced by abscisic acid (ABA) to reduce water loss by transpiration.[1] Stomata are epidermal structures distributed on the surface of leaves and stems. The opening and closing of the pore is mediated by turgor-driven volume changes of two surrounding guard cells. During drought plants accumulate ABA, which triggers in guard cells a signalling cascade that leads to stomatal closure.[2]

Engineering of stomatal activity is an attractive biotechnological target to reduce the water requirements of crops and to enhance productivity under stress conditions.[3,4] Simulation studies indicate that under drought, earlier and tighter stomatal closure would reduce desiccation and sustain yield stability.[5] Experimental evidence from model species demonstrates that stomatal closure can be effectively enhanced either by the disruption of negative regulators of ABA responses, or by the constitutive expression of positive regulators.[6-8] Yet, manipulation of genes as such in crops will likely result in undesirable side-effects on plant growth and productivity, in addition to drought tolerance, as they are expressed in many tissues and control several yield parameters.[3,9] Proper genetic engineering of stomatal responses requires guard cell-specific promoters to selectively manipulate ABA signalling components in guard cells, without affecting other plant responses to the hormone.

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We have recently employed a large scale gene trap screen to identify candidate guard cell-specific genes and promoters in Arabidopsis.[10] Gene trap lines, in which endogenous proteins are fused to reporter markers, have been successfully exploited to identify genes expressed in restricted domains of plant tissues.[11-13] Nearly 20,000 gene trap lines were screened for guard cell-specific expression of the β-*glucoronidase* (*GUS*) reporter gene. In total we recovered 5 lines in which the reporter was exclusively or preferentially expressed in guard cells.

In three lines insertion of the trapping element occurred in intergenic regions on chromosome 1, 2 and 5, respectively. We examined the expression of the genes surrounding the insertion site in laser-capture-microdissected guard cells and mesophyll cells.[14] Expression analyses identified two genes selectively expressed in guard cells, corresponding to a *PP2C PROTEIN PHOSPHATASE* and to an unknown *EXPRESSED PROTEIN* gene.[10] In the other two lines insertions occurred in two annotated genes, encoding for the CYTOCHROME P450 86A2 (CYP86A2) mono-oxygenase and the PLEIOTROPIC DRUG RESISTANCE 3 (AtPDR3) transporter, respectively. RT-PCR experiments in purified stomata confirmed the guard cell-specific expression of the trapped genes.[10] Examination of the five gene trap lines for unusual phenotypes revealed that the loss of the *AtPDR3* gene function impaired stomatal responses to ABA.[10] This finding establish a functional link between the guard cell-specific expression of the trapped gene and stomatal activity, and validate the use of gene trap screens for the identification of cell-specific genes and mutations.

In addition to gene discovery, gene traps have been successfully used for the identification of promoters that drive specific expression.[15] Thus, the lines described in our previous study represent a potential source of novel guard cell-specific promoters. To validate this assumption, we are currently isolating the genomic regions located upstream of the trapped genes for further analysis. As a proof of concept, here we report results from the characterization of the putative *CYP86A2* promoter.

The 1,256 bp chromosomal region upstream of the start codon of *CYP86A2* (At4g00360) was amplified by PCR and fused to the *sm-Green Fluorescence Protein* (*smGFP*) reporter gene.[16] The

transcriptional fusion (*pCYP:smGFP*) was introduced in Arabidopsis by *Agrobacterium*-mediated transformation.[17] 12 independent T2 *pCYP:smGFP* transformants were selected for the analysis of GFP patterns.

All the transgenic lines displayed strong GFP signals in guard cells of young and mature tissues (Fig. 1). Our previous examination of the *CYP86A2* gene trap allele, indicated that in young seedlings the endogenous gene is expressed in other cell types, in addition to stomata. Three-day-old gene trap seedlings disclosed diffused GUS staining in the epidermis of cotyledons and hypocotyls and in the upper part of the root vascular system.[10] Interestingly, transgenic *pCYP:GFP* seedlings showed guard cell-specific expression of the reporter, both in cotyledons and hypocotyls (Fig. 1A–C). Conversely, GFP patterns confirmed the guard cell-specific expression of *CYP86A2* in developing and fully expanded rosette leaves, previously revealed by GUS activity in the gene trap line (Fig. 1D).

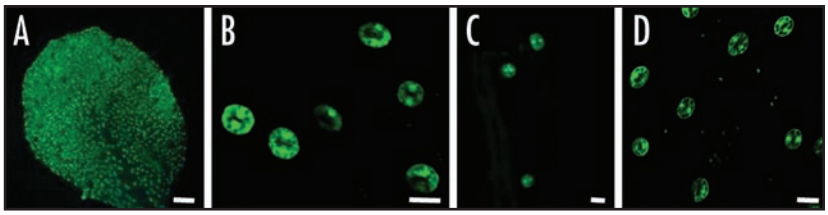

Figure 1. Optical and confocal analyses of GFP expression in the pCYP:GFP lines. (A–C) GFP expression in tissues of three-day-old seedlings; (A) cotyledon (scale bar 1 mm), (B) detail of a cotyledon (scale bar 20 μm), (C) hypocotyl (scale bar 20 μm). (D) GFP expression in a developing rosette leaf (scale bar 20 μm).

The discrepancy between GUS expression in GT1345 and GFP expression in *pCYP:GFP* seedlings could be due to positional effects associated to the insertion site of the transgene. Nevertheless, this hypothesis is very unlikely, as we consistently observed guard cell-specific GFP expression in seedlings from 12 independent transformation events. A more likely explanation for the enhanced guard cell-specificity, observed for the 1,256 bp genomic fragment compared to the endogenous promoter, is the lack of *cis*-regulatory elements that drive the expression of the *CYP86A2* gene beyond guard cells. Clearly more work is needed to accurately define the regulatory elements significant for *CYP86A2* expression.

Very few guard cell-specific plant promoters have been described. Among them the Arabidopsis *AtMYB60* promoter showed highly specific guard cell activity.[18] Yet, *AtMYB60* expression is rapidly downregulated by ABA and dehydration stress.[18] Constitutive or stress-inducible guard cell-specific promoters would be preferable for engineering stomatal responses to enhance plant drought tolerance. We analyzed the effect of ABA and desiccation on the expression of the endogenous *CYP86A2* gene in wild type plants. As shown in Figure 2, *CYP86A2* expression was slightly upregulated by ABA and dehydration treatments. These results are in agreement with microarray expression data compiled by Genevestigator, which show that ABA treatment, osmotic, oxidative and salt stress result in less than two-fold induction in gene expression of *CYP86A2*.[19]

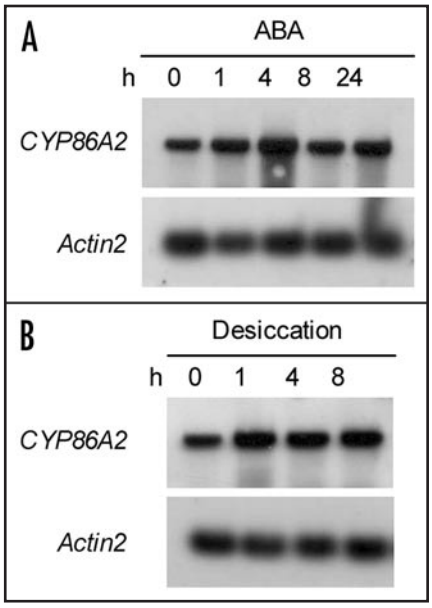

Figure 2. Analysis of CYP86A2 expression in response to ABA and desiccation. Three-week-old wild type plants were (A) treated with 100 μM ABA, or (B) dehydrated on Whatman paper for 8 hours at 21°C in the light. Total RNA samples were extracted at indicated time intervals, expressed in hours (h), and the *Actin2* gene was used as a control.

Data provided in this study indicate that the CYP86A2 promoter provides a novel tool for manipulating gene expression in guard cells, both for physiological studies and future biotechnological applications. We expect that the gene trap lines identified in our previous screen[10] will yield additional guard cell-specific promoters for the expression of target genes and reporters in stomata.

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