A 69 bp fragment in the pyrroline-5-carboxylate reductase promoter of *Arabidopsis thaliana* activates minimal CaMV 35S promoter in a tissue-specific manner

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Abstract **The *Arabidopsis thaliana* gene that encodes pyrroline-5-carboxylate reductase (*At-P5R*), the last enzyme in proline biosynthesis in *A. thaliana*, is developmentally regulated and is highly expressed in cells that divide rapidly or undergo changes in osmotic potential. A 69 bp region (P69; −120 to −51) has previously been identified in a 5′ deletion analysis of the *At-P5R* promoter to be necessary for the basal expression. Here, the essential role of P69 for tissue-specific expression of *At-P5R* is demonstrated by loss- and gain-of-function experiments.**

Key words: Gain of function; Loss of function; Proline

1. Introduction

Free proline accumulation has been reported in many higher plants in response to a wide range of environmental stresses, mainly osmotic ones. Several roles have been proposed for proline to counteract adverse growth conditions during stress [1]. In higher plants, free proline accumulation during osmotic stress is predominantly the result of enhanced biosynthesis from glutamate and reduced proline oxidation. The proline biosynthetic pathway from glutamate consists of two successive reductions, via pyrroline-5-carboxylate (P5C), catalyzed by the bifunctional enzymes P5C synthetase (P5CS) and P5C reductase (P5CR).

During plant development, the free proline content varies significantly in different tissues and at different developmental stages [2]. This variation is not due to the fluctuation in the total amino acid pool [3]. Proline accumulates preferably in organs that experience dehydration, such as floret and seeds [2], and can influence the plant development [4–11].

To understand the role of proline biosynthesis during development, we have analyzed the expression pattern of the *Arabidopsis thaliana* gene that encodes P5CR (*At-P5R*) with promoter-β-glucuronidase (*gus*) fusions. The expression of *At-P5R* is high in cells or tissues that experience developmentally programmed osmotic adjustment, such as guard cells, hydathodes, pollen grains and developing seeds [12]. Furthermore, *At-P5R* expression is also high in rapidly dividing cells, such as apical meristems, root meristems and lateral root primordia. We showed that a 69 bp fragment of the *At-P5R* promoter (P69) located between −120 and −51 with respect to the transcription start is necessary for the *At-P5R* tissue-specific expression [12]. We demonstrate here that P69 is necessary and sufficient to activate a minimal cauliflower mosaic virus (CaMV) 35S promoter in a tissue-specific manner.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *A. thaliana* (L.) Heynh. C24 were surface-sterilized and grown as described by Hua et al. [12].

2.2. Plasmid constructions

To perform P69 gain-of-function analysis, the 46 bp minimal CaMV 35S promoter and the 53 bp 35S leader were amplified by PCR from pJK4 [13] with the following primers: 5′-AAAACTGCAGGCCTCGCAAGACCCTTCCTCTATA (annealing to the 35S promoter) and 5′-AGCAATTGCCCGGCTTTCTTG (annealing to *gus*). The amplified fragment was digested with *Pst*I and *Nco*I and cloned in pGUS1 [12]. The recombinant plasmid was named pGUS-35sm and carried a fusion between a minimal 35S promoter (−46, +53) and *gus*.

To obtain P69, pGUS212 [12], which contains the *At-P5R* promoter (−120 to +219), was digested with *Hin*dIII and *Bsm*I. The fragment was further blunt-ended by T_4 DNA polymerase and cloned in the *Stu*I site of pGUS-35sm. The plasmids with two tandem copies of P69 in both the sense (p69-GUS4) and antisense (p69-GUS5) orientation were used in further experiments.

The loss-of-function analysis was performed with an *At-P5R* promoter in which P69 was removed. Briefly, two separate PCR amplifications were done with the 994 bp *At-P5R* promoter [12] as template by using primers 1 (5′-CGTCATCAACGATGCATCAGGA) and 2 (5′-CCTTCTGCGCGTGTAGGCTTTTGACTTGGGTTTG), and primers 3 (5′-CAAACCCAAGTCAAAAGCCTACACGCGCAGAAGGCTATA) and 4 (5′-GAATCTCCATGGGGAAAATTTAAC). Subsequently, the two PCR products were mixed and amplified with primers 1 and 4. The 368 bp amplified fragment with the deletion of P69 was digested with *Nsi*I and *Nco*I and was used to replace the same region of the *At-P5R* promoter in pGUS994 [12]. The resulting plasmid was named pLOF and contained the *gus* gene fused to the *At-P5R* promoter in which P69 (−120, −51) was deleted.

The binary vector pGSV4 [12] was used to transform *Arabidopsis*. The *Hin*dIII-*Sma*I fragments from the above constructs that included the chimeric *gus* gene and the nopaline synthase gene (*nos*) terminator were cloned into the *Xba*I (filled in with Klenow) and *Hin*dIII sites of pGSV4. The resulting binary vectors were designated pTiGUS-35sm, pTi69-GUS4, pTi69-GUS5 and pTiLOF.

2.3. Transformation of Arabidopsis

The above-mentioned binary vectors were transferred to *Agrobacterium tumefaciens* C58C1Rif^R via direct $CaCl_2$ transformation and *A. thaliana* was transformed as described [14]. The transgenic plants with the constructs were named 69-GUS4, 69-GUS5 and LOF.

2.4. Histochemical GUS assay of transgenic lines

Histochemical assays for GUS activity were performed overnight

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Abbreviations: CaMV, cauliflower mosaic virus; *gus*, β-glucuronidase; P5R, pyrroline-5-carboxylate reductase; P5CS, pyrroline-5-carboxylate synthase

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with 10 day old seedlings or 5 week old mature plants as described [12]. Approximately 20 independent transgenic lines were analyzed for each construct. Only two LOF, three 69-GUS5 and five 69-GUS4 lines showed detectable staining. Pictures were taken under bright field microscopy.

3. Results

In a previous study [12], a 339 bp region of *At-P5R* (−120 to +219 relative to the transcription start) was shown to confer tissue-specific expression of the *gus* reporter gene. Given that a further deletion to −51 resulted in a complete loss of basal expression, the 69 bp *At-P5R* region (−120 to −51), P69, is necessary for basal expression. In order to investigate whether P69 is sufficient to confer tissue-specific expression, gain- and loss-of-function approaches were carried out in transgenic *Arabidopsis*.

3.1. In silico analysis of P69

P69 shows sequences homologous to transcription factor-binding sites (Fig. 1). Sequence CAAAATAAG that stretches from position +3 to +11 resembled the *cis*-element from tobacco that is responsible for the production of a pollen-specific protein [15], but with a one base substitution in the hexamer core AAATGA. A TGA1b-binding site, TGACG, that is involved in the root-specific expression of the CaMV 35S promoter [16] was found from position +48 to +52 as well as a Myb-binding site at position +40 to +44 [17] on the opposite strand. The stress-inducible AtMYB2 protein from *Arabidopsis* [18] may regulate the expression of the P5CS gene, *At-P5S*, implying a role for Myb-like factors in proline biosynthesis. Matches to the binding site of homeo domain factors [19] and Ik2 factor [20], both involved in the differentiation of vertebrates, were located at positions +28 to +31 and +55 to +58, respectively. The role of homeo domain factors in plant development has also been documented [21,22].

3.2. Gain-of-function experiment: comparison of the expression patterns driven by the 994 bp At-P5R promoter or by P69

P69 was studied for its ability to confer regulated expression to *gus*. Two tandem repeats of P69 were inserted, either in the sense or in antisense orientation, in front of a minimal 35S promoter fused to *gus* (Fig. 2A). The constructs were stably transformed into *Arabidopsis*. Histochemical analysis was performed in 10 day and 5 week old plants (Fig. 3a–i). Localization of the GUS activity conferred by P69 or by the

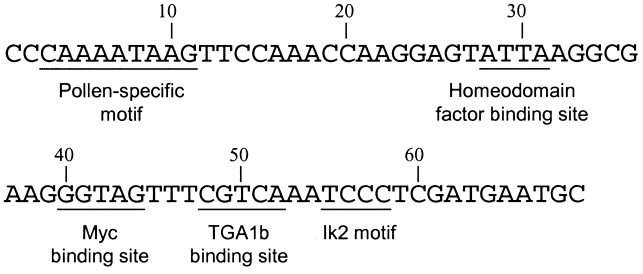

Fig. 1. Conserved DNA motifs in the *At-P5R* 69 bp fragment (−120 to −51). The sequences homologous to the binding sites of the transcriptional factors are underlined. The names of the motifs are indicated below the sequences and the numbers correspond to the positions in P69.

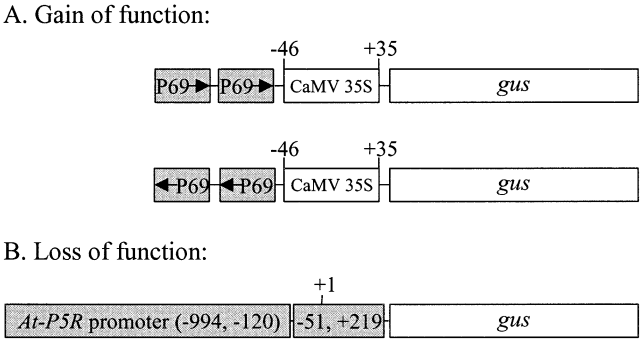

Fig. 2. Schematic representation of the gain-of-function and loss-of-function constructs. The constructs were made as described in Section 2. The sequence from the *At-P5R* promoter is represented as a solid box. The arrowheads and the numbers indicate the orientation of P69 and the positions in CaMV 35S or in the *At-P5R* promoter, respectively. The transcriptional start site of *At-P5R* is marked as +1. The binary vector is derived from pGSV4.

994 bp *At-P5R* promoter (observed in GUS994 lines [12]) is compared in Table 1. In the aerial part of 69-GUS4 seedlings, *gus* was expressed in emerging shoot primordia of apical meristems (Fig. 3a), hydathodes and young leaf blades (Fig. 3b) as it was in GUS994, although to a lesser extent. However, the expression in guard cells and leaf veins was seen in GUS994 only. In roots, 69-GUS4 mainly retained the expression pattern of GUS994, but the GUS staining was weaker. GUS activity could be detected in root meristems, lateral root primordia and vascular bundles (Fig. 3c and d) and in crowns (Fig. 3e). In adult plant, only pollen grains (Fig. 3f) exhibited clear GUS staining. Very weak staining was visible in developing seeds (data not shown).

69-GUS5 lines showed a similar but weaker expression in apical meristems (Fig. 3g), hydathodes (Fig. 3h) and a few pollen grains (Fig. 3i), but not in roots. No expression could

Table 1
Histochemical localization of GUS activity in GUS994, 69-GUS4 and 69-GUS5 lines

Cells or tissues	Transgenic lines		
	GUS994	69-GUS4	69-GUS5
10 day old plant			
Apical meristem	Y	Y	Y
Young leaf blade	Y	Y	N
Vein	Y	N	N
Guard cell	Y	N	N
Hydathode	Y	Y	Y
Base of trichome	Y	N	N
Root meristem	Y	Y	N
Lateral root primordia	Y	Y	N
Root vascular cylinder	Y	Y	N
Crown	Y	Y	N
Adult plant			
Leaf blade	Y	N	N
Vein	Y	N	N
Hydathode	Y	N	N
Guard cell	Y	N	N
Root tip	Y	N	N
Pollen grain	Y	Y	Y
Carpel and stigma	Y	N	N
Ovule	Y	N	N
Central septum	Y	N	N
Developing seed	Y	Y	N

Y, present; N, absent.

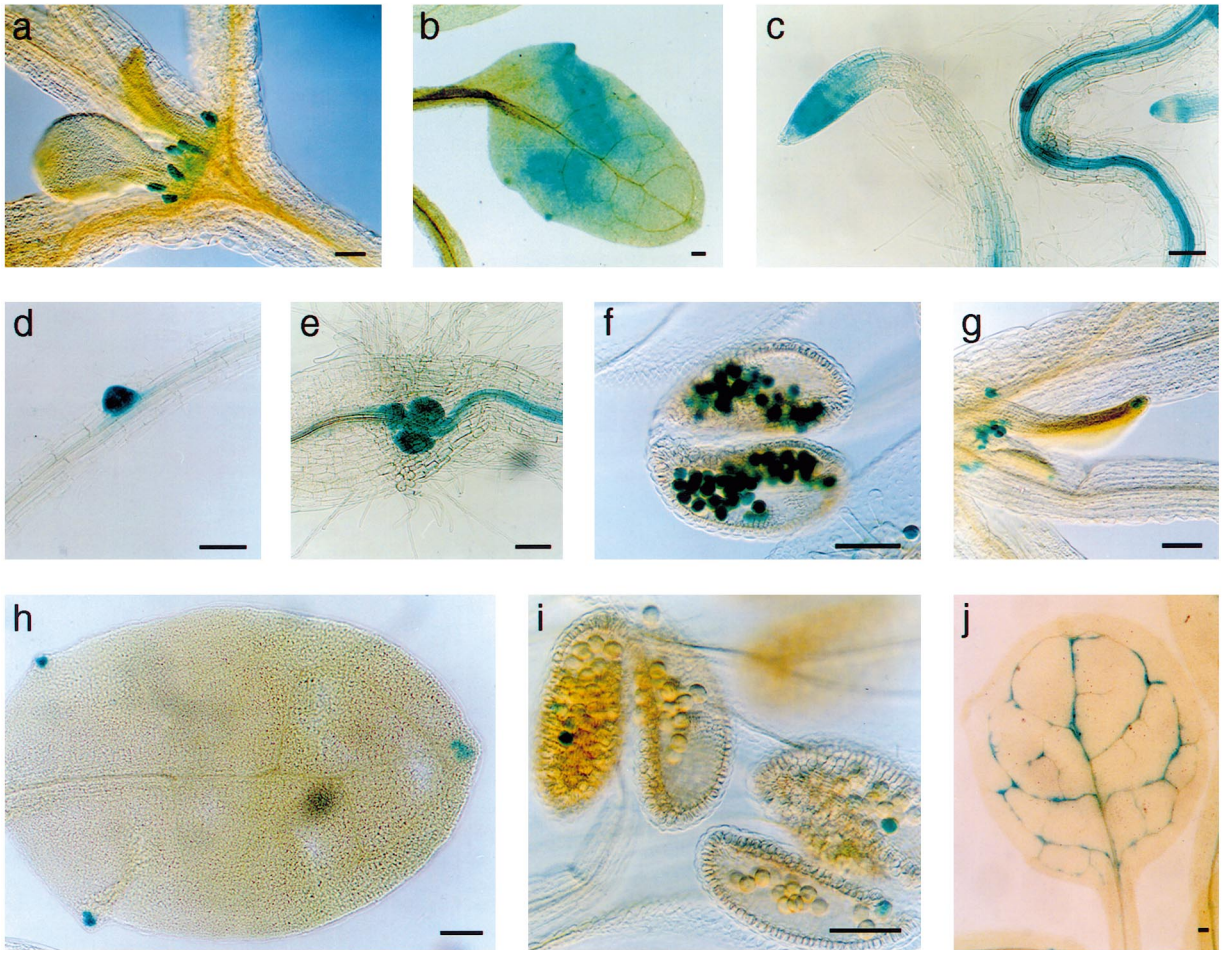

Fig. 3. Histochemical localization of the GUS activity in transgenic *Arabidopsis* 69-GUS4, 69-GUS5 and LOF plants. (a) Apical meristem, (b) young leaf blade and hydathode, (c) main root meristem, lateral root primordia and vascular bundles, (d) lateral root primordia and (e) crown of 10 day old seedlings of 69-GUS4 plants, (f) pollen grain of 5 week old adult 69-GUS4 plant. (g) Apical meristem and (h) hydathode of 10 day old seedlings of 69-GUS5 plants, (i) pollen grain of a 5 week old adult 69-GUS5 plant. (j) Leaf vein of a 10 day old seedling of LOF plant. Bars = 100 μm.

be detected when *gus* expression was driven by the minimal 35S promoter (data not shown).

3.3. Loss-of-function analysis

To investigate the redundancy of *cis*-elements involved in the tissue-specific *At-P5R* expression, a P69-deleted *At-P5R* promoter derivative was constructed (Fig. 2B). Transgenic plants with the LOF construct were analyzed (Fig. 3j). Reporter gene expression was only detected in leaf veins.

4. Discussion

To understand whether P69 is sufficient to direct the tissue-specific expression observed with the 994 bp *At-P5R* promoter [12], P69 was fused upstream of a minimal CaMV 35S promoter. A 46 bp minimal 35S promoter was used to avoid possible influences of the elements in longer 35S promoter, because between −90 and −46, elements have been reported that can interact with upstream ones to direct expression in leaves [23].

The minimal 35S promoter alone does not direct any *gus* expression. However, when fused to P69, the chimeric 35S promoter confers regulated expression to the reporter gene.

Our results demonstrate that P69 is sufficient for expression in apical meristems, root meristems, lateral root primordia, root vascular bundles, hydathodes, young leaf blades, crown, pollen grains and developing seeds. Putative pollen- and root-specific motifs were indicated by in silico analysis. However, P69 does not confer any detectable expression to the reporter gene in guard cells, base of trichomes, ovules, septum and leaf veins, where *At-P5R* is also expressed. Three explanations for these results can be proposed. First, the expression may be too weak to be detected. Second, a stringent spacing between P69 and the TATA box might be required for its functionality in specific tissues. Finally, P69 may be not sufficient to mimic the complete tissue-specific expression of *At-P5R*. *Cis*-regulatory elements outside P69 might be required for the integral tissue-specific expression of *At-P5R*. Given that the *At-P5R* promoter still showed complete tissue-specific expression after deletion to −120 bp, the 51 bp sequence between P69 and the transcription start site or the leader may still contain important elements. Leaders have previously been reported to participate in gene transcription [24,25].

The orientation of the 69 bp fragment is important for expression in some, but not all, tissues. The *cis*-acting elements responsible for the expression in apical meristems and

hydathodes can function in both orientations. On the contrary, the regulatory elements for young leaf blade, crown and root-specific expression function only in the sense orientation. In pollen grains, the *cis*-elements function better in the sense than in the antisense orientation.

Taken together, these results and those published previously [12] indicate that *At-P5R* expression in leaf veins depends on the presence of *cis*-elements outside P69. On the other hand, elements within P69, which are responsible for the expression in other tissues, are most probably not redundant and may interact with other *cis*-acting elements for expression in guard cells, base of trichome, septum, stigma and vascular bundle of filament.

Short promoter sequences have been reported to drive tissue-specific gene expression. A 143 bp sequence of the *APETALA3* promoter can confer expression to the reporter gene in petals and stamens as does the full promoter when fused with a minimal CaMV 35S promoter. This 143 bp sequence has to be repeated three times to be functional [26]. A monomer of a 30 bp fragment of the 'late anther tomato' (*LAT52* and *LAT59*) gene promoter is necessary and sufficient to direct pollen-specific expression in a transient expression assay [27]. However, in the above-mentioned two examples, the genes are expressed predominantly in one particular organ. Therefore, it is unclear how a complex tissue specificity can be regulated by a relatively short promoter sequence. A 140 bp promoter fragment of the osmotin gene is necessary for the tissue-specific expression, but its sufficiency to direct a minimal 35S promoter was demonstrated in a transient expression assay [28]. Therefore, the tissue-specific expression conferred by this 140 bp fragment still awaits analysis in transgenic plants. In short, this work establishes that the tissue specificity of *At-P5R* is mainly associated with P69.

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