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Light regulation of a SSRubisco-*nos* chimaeric gene: photoregulatory control sequences from a C_3 plant function in cells of a CAM plant

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ABSTRACT

A SSRubisco-nos chimaeric gene has been constructed, in an oncogenic Ti-plasmid vector. A 900bp soybean SSRubisco upstream fragment, carrying CAAT and TATA boxes and transcription initiation point, was fused to the nos coding region, the fusion site being within the 5'-untranslated region. When this chimaeric construct was transferred to Kalanchoe cells, nos expression was shown to be light-regulated. Thus DNA sequences responsible for light-dark control of gene expression are wholly or partly contained within the 900bp soybean SSRubisco upstream region. Moreover, this is the first demonstration that photoregulatory elements in a gene derived from a C_3 plant, function in cells of a plant exhibiting the CAM trait.

INTRODUCTION

Ti-plasmid gene-transfer technology has been utilised to identify DNA sequences mediating light regulation of gene expression. Such elements have been found in the upstream regions of genes encoding the small subunit of ribulose 1,5 bisphosphate carboxylase (SSRubisco)[1-4], the chlorophyll a/b binding protein[5,6] and chalcone synthase[7]. Photoregulation of SSRubisco involves an upstream enhancer which acts bidirectionally[4], although additional sequences may be necessary[3]. Tissue specificity[8] is also conferred by the upstream region[9]. In addition to its enzymic activity, the SSRubisco coding region contains sequences specifying chloroplast localisation[10,11].

Its many interesting features make SSRubisco a challenging intellectual problem. It is also a potential donor of DNA sequences useful in gene-transfer approaches to crop improvement. Thus far, SSRubisco analyses have been limited to transfers between C_3 plants. However, in plants exhibiting C_4 or Crassulacean Acid Metabolism (CAM) photosynthetic physiology is subtly different: in C_4 plants, SSRubisco expression is light-regulated, but occurs only in bundle sheath cells[12]; in both C_4 and CAM plants, primary fixation of CO_2 occurs via carboxylation of phosphoenolpyruvate[13]; in CAM plants this process occurs in the dark.

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Many C_4 and CAM plants are of agronomic significance. It is thus of importance to ascertain whether the regulation of genes from C_3 plants is affected by the differences shown by these plants. Accordingly we have commenced a study of the photoregulation of C_3 genes in C_4 and CAM plants. Here we report the construction of a chimaeric gene in which SSRubisco[14,15] upstream sequences derived from a C_3 plant, soybean (Fig. 1), have been fused to the nopaline synthase (nos)[16] coding region. Upon transfer to cells of the CAM plant, Kalanchoe daigremontiana, this construct is photoregulated.

MATERIALS AND METHODS

Microbiological Techniques

Bacterial growth conditions, antibiotic concentrations and DNA purifications were as previously described[17].

General Cloning Procedures

Conditions for restriction enzyme digestion, linker manipulations, ligations and transformation were as described[17]. DNA fragment isolation was by freeze-elution[18]. Transformants were screened by colony hybridisation[19] using nick-translated probes[20] and mini-lysate plasmid purification[21].

Genetic Procedures

Transmission of pGV1106 derivatives from E.coli to A.tumefaciens was mediated by the IncN plasmid pRN3[17,22]. Selection in A.tumefaciens of recombinants between the transmitted plasmids and resident Ti-plasmids was as previously described[16,17]. Putative recombinant Ti-plasmids were screened by Southern blotting of total A.tumefaciens DNA[23].

Botanical Techniques

Tumour induction on stems and leaves of Kalanchoe daigremontiana, nopaline synthase assays[25,26], and culture of axenic tumour tissue was by previously published methods[16,17,24]. Conventional light/dark regime utilised fluorescent lighting in a 16hr light/ 8hr dark cycle at 25°C.

RNA Isolation and Dot Blotting

Total RNA was isolated from axenic callus tissue[27], dot blotted onto nitrocellulose[28] and probed with nick translated DNA[20].

RESULTS

The Acceptor Plasmid

The acceptor plasmid pDUB1111 (Fig. 2), in which the SSRubisco-nos gene was assembled, is a product of a deletion strategy which resulted in a functional map of the nos promoter and the T-DNA right border [16,24]. pDUB1111

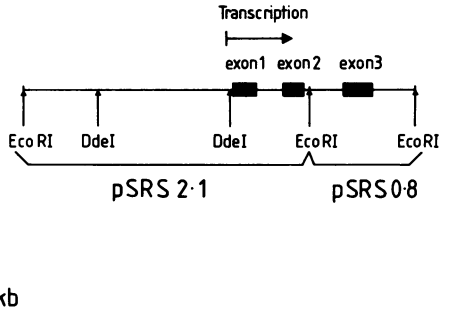

Fig. 1 Structure of Soybean SSRubisco[14], showing Eco RI fragments subcloned in pSRS2.1 & pSRS0.8. Only Dde I sites bracketting fragment described in text are shown.

is derived from pASK1029[17], a pBR322 derivative containing Hin dIII fragment 23 (HIII-23) of pTiC58, a 3.2Kb fragment spanning the right border of the T-DNA. pDUB1111 was constructed via Bal 31 deletion of sequences between -1 and -287 in the nos upstream region (numbered as ref 29). This deletion (Fig. 3c) removes the CAAT and TATA boxes from the nos promoter[14], replacing them with an Eco RI site, but retains the nos coding region and the right T-DNA border 25bp repeat[24]. This deletion, recombined into pTiC58 to form pDUB1006, has no effect on oncogenicity[24], but completely abolishes nos promoter activity[14]. pDUB1111 is thus a general purpose acceptor plasmid, carrying a promoter-deleted nos gene, with a convenient restriction site immediately upstream, into which putative promoter/regulatory elements may be inserted. The activity of these elements may then be assessed quickly and easily by the appearance of nopaline synthase in tumour tissue[14]. This preliminary rapid analysis can be confirmed by direct RNA techniques.

Construction of SSRubisco-nos chimaeric gene

Dr R. Meagher (University of Georgia USA) kindly made available to us the soybean SSRubisco gene[14], the structure of which is shown in Fig. 1. The gene is present in two pBR325 derivatives: the 5' part (pSRS2.1) includes upstream sequences and the first two exons; and the 3' part (pSRS0.8) includes the third exon and downstream sequences (Fig. 1).

Centred around a position 12bp 5' of the ATG codon is a Dde I site, with the nearest similar site approx. 900bp upstream[14]. Dde I thus delineates a fragment containing about 900bp of upstream sequence, including the putative CAAT, and TATA boxes, the transcription initiation point, and (when flush) 38bp of the 5' untranslated region (Fig. 3e). This fragment from

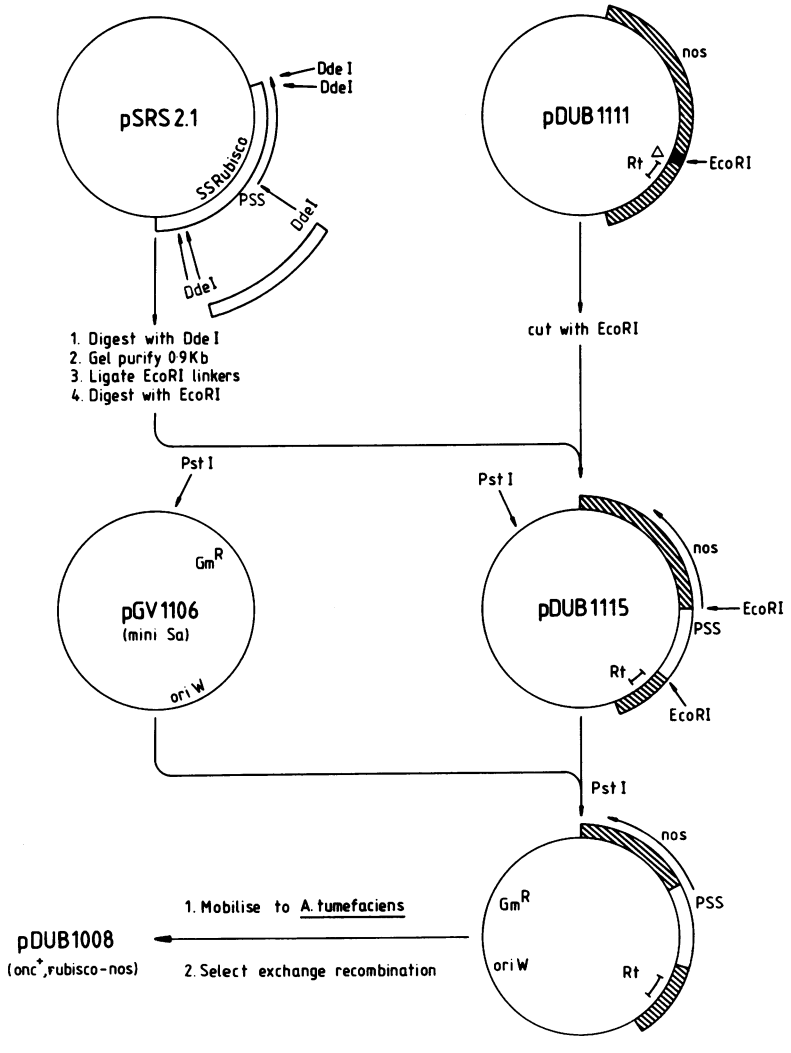

Fig. 2 Construction of SSRubisco-nos chimaeric gene. See text for details. Hatched box: Hin dIII fragment 23 of pTiC58; open box: SSRubisco; Rt: right border; PSS: promoter fragment of SSRubisco; oriW: IncW plasmid origin of replication; Gm^R: gentamycin resistance.

pSRS2.1 was gel purified, and its ends rendered flush with DNA polI (Fig. 2). After ligation of Eco RI linkers, the fragment was inserted into Eco RI-cut pDUB1111. Transformants carrying the insert, were identified by colony hybridisation using the 900bp fragment as probe, and the correct insert

Fig. 3 Nucleotide sequences concerned with SSRubisco-*nos* chimaeric gene. Sequence motifs involved in transcription[16] and T-DNA transfer[24] aligned for clarity. (a) Wild-type *nos*$^+$ gene[16,29], pGV3105. (b) *nos*$^+$ deletion-insertion mutant[16], pDUB1003^31. (c) *nos*$^-$ deletion in pDUB1006 and pDUB1111. (d) SSRubisco-*nos* chimaeric gene in pDUB1008 and pDUB1115. (e) Wild-type SSRubisco in pSRS2.1, extending to upstream Dde I site[14].

orientation checked by mini-lysate. A plasmid with the correct SSRubisco-*nos* chimaeric gene was named pDUB1115 (see Figs. 2, 3d & 4). A plasmid carrying the reverse orientation of the SSRubisco insert, named pDUB1120, was also picked for further analysis.

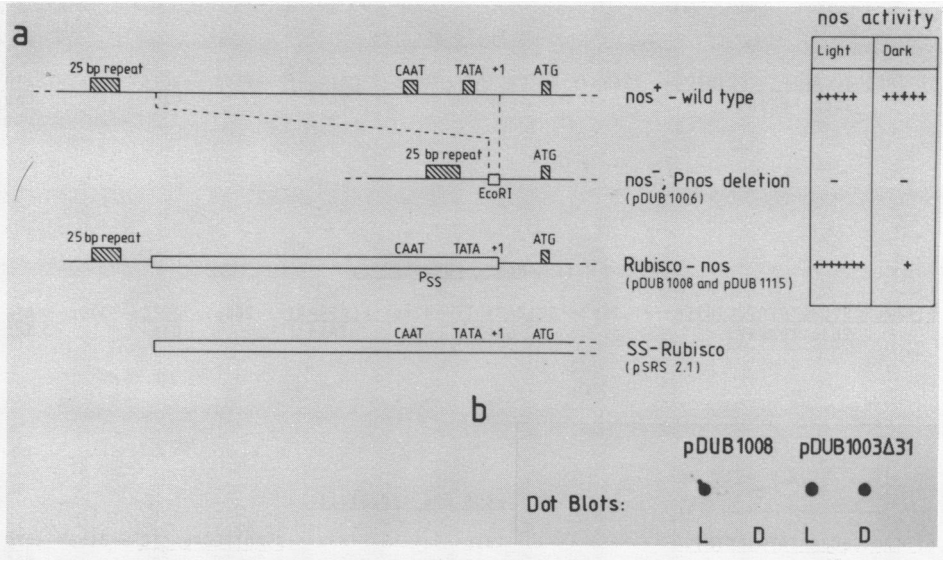

Fig. 4 (a) Summary of structures of Ti-plasmids considered in text, shown alongside nos assay results. Data presented for: nos+ (pGV3105 & pDUB1003^31); nos- (pDUB1006 & pDUB1010); SSRubisco-nos (pDUB1008). nos assays performed on tumours excised before (Light) or after (Dark) a prolonged (7-9 days) shift to dark conditions. Results represent averages determined from a minimum of six different inoculation sites. +++++ = 100% of wild-type nos+ activity in light. (b) Dot-blots of total RNA purified from axenic tumour tissue induced by pDUB1003^31 or pDUB1008, before (L) or after (D) 5 day shift to dark conditions, probed with nos coding region fragment.

To Pst I cleaved pDUB1115, was ligated similarly cut pGV1106, an IncW broad host range replicon[38]. Following pRN3 mediated transmission[22] to A.tumefaciens C58C1(pDUB1006) exchange recombination resulted in pDUB1008, a fully oncogenic Ti-plasmid, with the SSRubisco-nos hybrid gene close to, and internal of the T-DNA right border. Similar treatment of pDUB1120, resulted in an almost identical plasmid, pDUB1010, with the reverse orientation of the SSRubisco fragment relative to the nos coding region.

Expression from the SSRubisco-nos chimaeric gene

Duplicate tumours were induced on stems and leaves of several Kalanchoe daigremontiana plants using isogenic A.tumefaciens strains harbouring one of the following derivatives of pTiC58: pGV3105, the progenitor of the Ti-plasmids described here, which has a wild type phenotype with regard to oncogenicity and nos (Fig. 3a) ; pDUB1003^31, phenotypically almost indistinguishable from pGV3105[14,24], which has a deletion from -130 to

-302 in the nos upstream region replaced by a 1.2Kb insert (Fig. 3b); pDUB1006, a nos$^-$ onc$^+$ derivative resulting from the recombination of pDUB1111 into pGV3105 (Fig. 3c); pDUB1008, carrying the SSRubisco-nos chimaeric gene (Fig. 3d); and pDUB1010, possessing the reverse orientation of the SSRubisco fragment relative to the nos coding region.

After 4-6 weeks incubation in planta under a light/dark regime, nos assays were performed on tumours excised from parts of the plants. The plants were then shifted into dark conditions for 7-9 days before nos assays were performed on the remaining excised tumours.

Tumours induced by pGV3105 and pDUB1003^31 displayed no evidence of light-regulation (Fig. 4a) yielding high amounts of nos activity regardless of light presence. pDUB1006 and pDUB1010 induced tumours with no detectable nos activity under any light regime. These data show that removal of sequences between -1 and -287 from the nos upstream region results in promoter deletion, and that the inverse orientation of the SSRubisco fragment does not provide an active promoter configuration. In contrast, in tumours induced by pDUB1008, nos levels up to 50% higher than pDUB1003^31 were detected in light-grown tissue. However, after the shift to dark conditions, nos activity declined to about 20-30% of that found in the nos$^+$ strains.

The above results provided a preliminary indication that the soybean SSRubisco promoter was functional in Kalanchoe, and that at least some form of photoregulation was operative. To confirm this result, axenic tumours from K.daigremontiana were cultured in vitro under a light/dark regime. Each tissue was then split into two parts, total RNA being prepared from one part[27], and the other part transferred to dark conditions. After 5 days incubation in the dark, total RNA was prepared from the other part. These RNA preparations were dot-blotted onto nitrocellulose, and probed using a fragment from the nos coding region.

Under light conditions, callus tissue induced by both pDUB1008, and pDUB1003^31 had detectable and equivalent amounts of nos transcripts (Fig. 4b). However in the same tumours transferred to the dark, nos RNA was almost undetectable in pDUB1008 tissue, but remained detectable at a reasonably high level in pDUB1003^31 tissue.

Taken together these results demonstrate that the photoregulated soybean SSRubisco promoter is active in cells from a CAM plant. The results also indicate that the nos coding region contained within pDUB1111 can be used as a reliable indicator of activity of an inserted promoter.

DISCUSSION

The results presented here demonstrate that the soybean SSRubisco promoter is active and light-regulated in Kalanchoe. The inference of this is that the elements wholly or partly responsible for photoregulation are located between approx -900 and +38 in the soybean SSRubisco upstream region, consistent with previous observations[18].

This is the first demonstration of light-regulation of a gene from a C_3 plant in cells of a CAM plant. In contrast to C_3 and C_4, little is known about regulation of Rubisco in CAM plants. Primary CO_2 fixation takes place in the dark, via carboxylation of phosphoenolpyruvate[13]. Decarboxylation and Rubisco-mediated fixation into carbohydrate occur in the light[13]. Unlike C_4 plants, all chloroplasts in leaves of K.daigremontiana contain Rubisco[13]. The results presented here may shed some light on photoregulated gene expression in CAM plants.

Photoregulation of SSRubisco is controlled by phytochrome[31-33], at least in part at the level of transcription[34,35]. Whatever mediates the signal transmission from phytochrome to light-regulated genes in Kalanchoe is capable of activating a soybean promoter. In view of the differences in photosynthetic physiology of these plants, this indicates that the factors involved in photoregulation, are conserved despite wide evolutionary separation.

The SSRubisco-nos construct retains the correct spacing between the TATA box and the normal SSRubisco transcription initiation point, but also places the usual nos cap site at +49 in the SSRubisco-nos 5'-untranslated (UT) region. This results in an increase in the length of 5'-UT in SSRubisco-nos to 83bp from 36bp (nos[29]) or 47bp (SSRubisco[14]). The presence of secondary structure in the 5'-UT may reduce translational efficiency[8,36], and our construction positions an Eco RI site at +42. Experiments are underway to determine the transcription initiation point of the chimaeric gene, and whether these changes influence photoregulation.

Overtly, the fact that light-regulation of a chimaeric gene can be detected by a simple nos assay is a surprising result. However these assays were performed on non-axenic tumours, probably containing the inciting Agrobacterium. Thus nopaline would be consumed in the absence of further synthesis in the dark, resulting in the observed results. Previous workers have demonstrated that opine assays can be used as a reliable indicator of transcriptional activity[8,14,37,38], which our results confirm. Interestingly, when assessed on the relative amounts of tissue required to give a positive

signal, nos assays are some 20-100 times more sensitive than dot-blots as an arbiter of gene expression.

This paper represents the first stage in a long term investigation of the photoregulation of genes in CAM and C_4 plants. The next steps are to locate the DNA sequences involved, and to clarify the question of tissue specificity.

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