The tomato *RBCS3A* promoter requires integration into the chromatin for correct organ-specific regulation

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Abstract In tomato, the *RBCS1*, *RBCS2* and *RBCS3A* genes, encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, are expressed in leaves and light-grown seedlings, but only *RBCS1* and *RBCS2* are expressed in developing tomato fruits. The activities of the three promoters have been compared in transgenic plants and after transient transformation. Fruit-specific repression of the *RBCS3A* promoter was observed in transgenic plants, but not after ballistic transient transformation, indicating that chromatin integration is necessary for its correct organ-specific regulation. In addition, matrix attachment regions have been identified in the *RBCS1*, *RBCS2* and *RBCS3A* promoters. This is the second case in plants of absence of correct regulation of a plasmid-borne plant promoter and correlating potential nuclear matrix attachment of the gene.

Key words: Chromatin; Matrix attachment region; Organ-specific; *RBCS* promoter; Tomato

1. Introduction

The family of *RBCS* genes encodes the small subunit of ribulose-1,5-bisphosphate carboxylase, the key enzyme in photosynthetic carbon dioxide fixation. In tomato, the *RBCS* gene family consists of five members (*RBCS1, RBCS2, RBCS3A, RBCS3B,* and *RBCS3C*) at three chromosomal loci [1]. The mRNAs of all five *RBCS* genes accumulate to similarly high levels in leaves and light-grown cotyledons. In contrast, in young tomato fruits, only the *RBCS1* and *RBCS2* mRNAs accumulate to significant levels [2]. These differences in mRNA accumulation reflect different activities of the individual *RBCS* promoters in the various organs [3].

As part of our attempt to investigate the differential regulation of the tomato *RBCS* genes in young fruits, we have analyzed the activity of select promoters in tomato leaves and young fruits both in transgenic plants and after transient transformation. Surprisingly, we found that while the *RBCS3A* promoter is strictly repressed in the fruits of transgenic plants carrying a *RBCS3A*-β-glucuronidase gene (*GUS*) fusion, the identical promoter fragment is released from fruit-specific repression when used to transiently transform fruits. These data suggest that integration of the *RBCS3A* promoter into the chromatin is required for its proper regulation. A similar finding has been reported for the bean β-phaseolin gene, which is released from seed-specific expression after transient transformation [4]. This gene was found to be flanked by matrix attachment regions (MARs), specific DNA fragments responsible for the attachment of chromatin loops to the nuclear matrix [5]. Promoter methylation, nucleosomal structure, and nuclear matrix attachment are just three of several factors which can influence the expression of genes in their native chromatin context [6–8]. To begin an investigation into which factors might act on the tomato *RBCS* genes, we tested the promoter fragments of *RBCS1*, *RBCS2* and *RBCS3A* in nuclear matrix binding assays. We found that all three promoter fragments possess nuclear matrix-binding activity. In addition, the recently isolated plant nuclear matrix protein MFP1 [9] specifically binds to all three fragments.

2. Materials and methods

2.1. Plant material

Tomato (*Lycopersicon esculentum*) cultivar VFNT cherry LA 1221 was grown under greenhouse conditions. Leaflets from young, fully expanded leaves and young green tomato fruits (5–8 mm in diameter) were harvested and surface sterilized as described [10]. Transgenic tomato cultivar T5 plants have been previously described [11].

2.2. Reporter gene constructs

The plasmid pRTL2-GUS [12] was used as a reference construct and consists of the CaMV 35S promoter upstream of the tobacco etch virus leader fused to the *GUSA* gene of *Escherichia coli* (here referred to as 35S-GUS). For construction of RBCS2-LUC and RBCS3A-LUC, the 741 bp *Hin*dIII-*Nhe*I fragment from *RBCS2* and the 1 kbp *Hin*dIII-*Nhe*I fragment from *RBCS3A* [2] were fused in a translational fusion to a promoterless LUC-NOS 3′ construct in pUC119 [10].

2.3. Particle bombardment

Surface sterilized leaves were directly placed on agar plates containing 0.8% agar in H_2O. Fruits were cut into 0.5–1.0 mm thin slices and soaked for 5 min in CPW12 (CPW salts [13] supplemented with 12% (w/v) mannitol and 20 mM MES pH 6.0 [14]). Three slices per plate were placed on 0.8% agar in CPW12. 2 mg gold particles (Hereus, 0.4–1.2 μm diameter) were coated with a 1:1 ratio of RBCS-LUC reporter and 35S-GUS reference plasmid (3 μg each) essentially as described [10]. The plant material was bombarded with 5 μl (167 μg) DNA-coated gold particles by a helium driven Biolistic PDS 1000 System (Bio-Rad Laboratories, Hercules, CA) with 27 mm Hg vacuum. The distance between rupture disk and macrocarrier was 4.5 cm (level 2), the distance between macrocarrier and sample 4.0 cm (level 4). Rupture disks (Bio-Rad Laboratories) of 1800 psi for fruits and 1550 psi for leaves were used. Fruit slices were bombarded a second time after flipping the disks on the plate. The bombarded tissue was incubated for 20 h at room temperature.

2.4. Luciferase and GUS assays

The plant tissue was weighed, frozen in liquid nitrogen, pulverized in a mortar, and homogenized in extraction buffer EGL (0.1 M KPO_4 pH 7.8, 1 mM EDTA, 5% glycerol, 10 mM DTT), using 1 ml/g tissue for fruits and 2 ml/g tissue for leaves, with a PT 1200 Polytron (Kinematica, Switzerland). The extract was cleared twice by centrifugation (15 000 rpm, 10 min, 2°C). Protein concentration in the supernatants was determined with the Bio-Rad protein assay kit (Bio-Rad Laboratories). Luciferase activity was determined immediately after extract preparation using the Promega luciferase assay system (Promega,

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Madison, WI) and the light emission was measured for 1 min in a Lumat LB9501 luminometer (Berthold, Wildbad, Germany). GUS assays were performed as described [10].

2.5. Nuclear matrix binding assays

Nuclear matrix binding assays were performed essentially as described previously [8].

2.6. 'South-western' blot experiments

Histidine-tagged MFP1 was expressed in *E. coli* as described previously [9]. Total protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed for binding of radioactively labeled DNA fragments, as described previously [15]. Binding reactions contained 10 ng/ml labeled DNA fragments ($0.5–1.0\times10^6$ cpm/ml) and 10 μg/ml sheared *E. coli* genomic DNA as nonspecific competitor.

3. Results

Transgenic plants harboring a fusion of either 3 kbp of the *RBCS1* promoter, 0.6 kbp of the *RBCS2* promoter or 1 kbp of the *RBCS3A* promoter to *GUS* were analyzed for GUS activity in young leaves and young tomato fruits (5–8 mm in diameter) (Fig. 1A). Whereas in leaves all three promoters gave rise to approximately the same GUS activity, the GUS activity in fruits of *RBCS3A-GUS* plants was about 50-fold lower than the activity of *RBCS2-GUS* plants [11], consistent with the activities of the endogenous promoters [3].

Fig. 1B shows the results of ballistic transient transformation experiments in which the same promoter fragments as used in Fig. 1A were fused to the firefly luciferase reporter gene (*LUC*). For these experiments only the promoters with the highest and lowest activities in fruit (*RBCS2* and *RBCS3A*, respectively) were chosen. The constructs were co-transformed with a *35S-GUS* construct by ballistic transformation into leaves and young tomato fruits and activities are presented as LUC/GUS activity in order to normalize for transformation efficiency. Both in leaves and in young fruits, the *RBCS3A*-promoter-derived activity was about 40% of *RBCS2*-promoter-derived activity. Hence, the *RBCS3A* promoter has equal activity in leaves and young fruits, when assayed in a plasmid-borne state. The generally lower activity of the *RBCS3A-LUC* construct in both organs might be due to a different influence on LUC activity of the portions of *RBCS2* and *RBCS3A* coding regions fused to the luciferase reading frame [1]. This might not be the case for the GUS protein, which is known to tolerate diverse N-terminal fusions [16]. Alternatively, a weak enhancement of the *RBCS3A* promoter or a weak silencing of the *RBCS2* promoter in leaves might occur only when integrated into the chromatin. That the observed expression of the *RBCS3A-LUC* construct in young fruits was not due to potential wound inducibility of the *RBCS3A* promoter was shown by using fruits from transgenic *RBCS3A-GUS* plants in bombardments with uncoated gold particles. No increase of GUS activity due to bombardment was found in such experiments (data not shown).

The discrepancy between the results from stable and transient transformation implies that integration into the chromatin might be required for the correct organ-specific regulation of the *RBCS3A* promoter. Recently, several aspects of chromatin structure have been considered to have an influence on gene expression [6,7,17], one of them being the attachment of chromatin to the nuclear matrix [8]. We tested whether matrix attachment regions are localized in the three promoter fragments used for the expression analysis, in order to establish whether there is a potential for matrix attachment to be involved in the observed expression phenomenon. End-labeled restriction fragments from plasmids containing the promoter fragments indicated in Fig. 3A were mixed with tobacco nuclear matrix preparations in the presence of restricted plant genomic DNA as a nonspecific competitor, as previously described [8]. After incubation under binding conditions, bound and unbound DNA fragments were separated by centrifugation, and analyzed by agarose gel electrophoresis along with the input DNA. As controls, the plant MAR DNA fragment ToRb7-6Sca/Cla, and a plant DNA fragment known not to bind to the nuclear matrix were used [8]. Fig. 2 shows that the 3 kbp *RBCS1* promoter fragment, the 0.6 kbp *RBCS2* promoter fragment and the 1 kbp *RBCS3A* promoter fragment are specifically bound by the nuclear matrix. In addition, a shorter 0.7 kbp deletion fragment of the *RBCS1* promoter was also shown to be bound. No binding was found for the negative control fragment and for the vector fragments

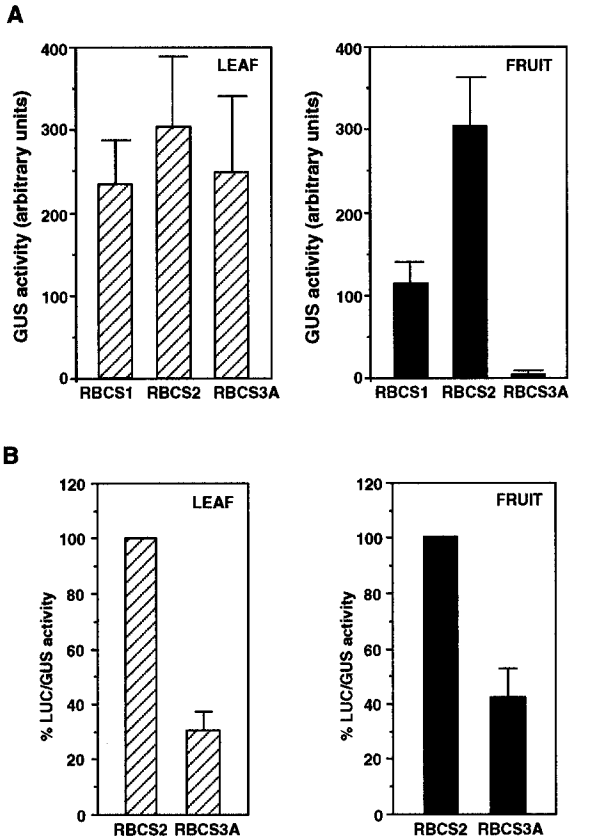

Fig. 1. Activity of *RBCS* promoters after stable and transient transformation. A: Fluorometric determination of GUS activity from leaves and young fruits (3–8 mm diameter) of transgenic plants containing translational fusions of the *RBCS* promoter fragments shown in Fig. 3A with *GUS* [11]. The mean values and standard deviations of six (leaves) and seven (fruits) samples from two to five different transgenic plants are shown. B: Activity of the *RBCS2* and *RBCS3A* promoters after transient transformation. Promoter fragments shown in Fig. 3A were fused to *LUC* in translational fusions and assayed after ballistic transient transformation together with a *35S-GUS* reference construct in leaves and young tomato fruits as described in Section 2. Data are expressed as % LUC/GUS activity, setting the activity derived from the *RBCS2-LUC* construct at 100%. Mean values and standard deviations of six individual transformations are shown.

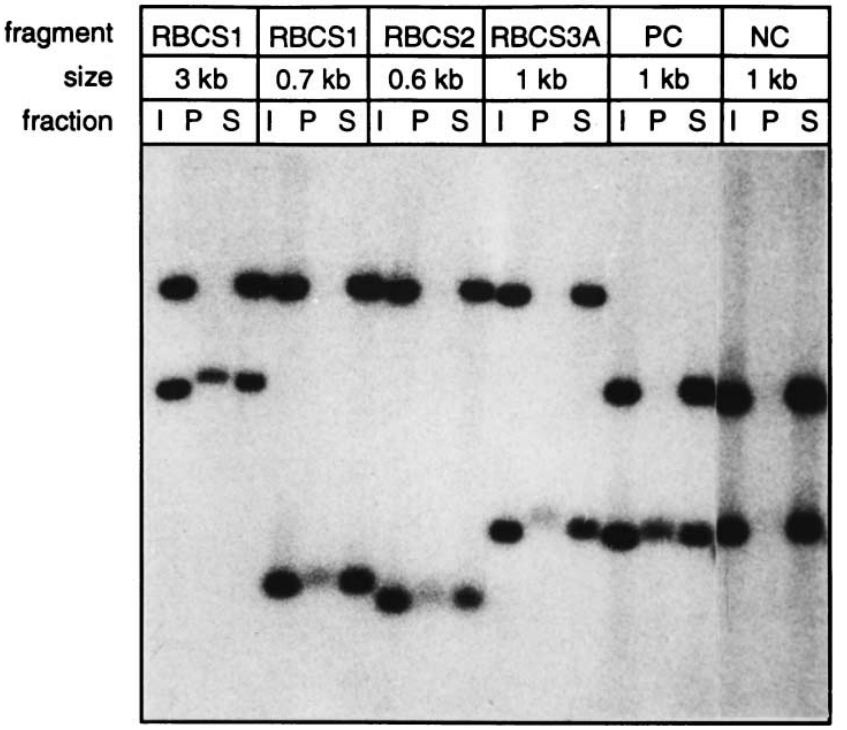

Fig. 2. Autoradiography of a gel showing a matrix binding assay of *RBCS* promoter fragments with a nuclear matrix preparation from tobacco NT-1 suspension cultured cells. The *RBCS1* 3 kbp fragment, the *RBCS2* 0.6 kbp fragment and the *RBCS3A* 1 kbp fragment are the *Hin*dIII-*Nhe*I fragments shown in Fig. 3A. The 0.7 kbp *RBCS1* fragment represents the downstream 0.7 kbp of the 3 kbp fragment shown in Fig. 3A. All *RBCS* fragments were released from the LUC-NOS 3′ transient transformation vector [10] by digestion with *Hin*dIII and *Nhe*I. I, input DNA; P, matrix bound fragments (pellet); S, unbound fragments (supernatant); PC, positive control (ToRb7-6Sca/Cla MAR in pBluescript) [8]; NC, negative control (non-MAR plant DNA fragment in pBluescript).

present in each reaction as internal controls. These data indicated that all three *RBCS* promoter fragments contain at least one matrix attachment region.

Recently, a nuclear matrix-localized protein MAR-binding from plants (MFP1) has been cloned [9]. In addition to the crude tobacco nuclear matrix fraction, we also tested this protein for binding to the three *RBCS* promoters. A truncated version of MFP1 shown to be sufficient for specific MAR binding [9] was expressed in *E. coli*, and the *E. coli* protein extract was separated by SDS-PAGE (Fig. 3B). Strips of protein blots were then incubated in 'South-western' reactions with the end-labeled *RBCS* promoter fragments shown in Fig. 3A. Strong binding of MFP1 to all three fragments was obtained. As a control, a MAR DNA fragment and a non-MAR DNA fragment from the *Drosophila* genome, previously used to characterize the binding specificity of MFP1, were used [9,15]. These data provide a second independent assay demonstrating the presence of matrix attachment sequences in the three *RBCS* promoters.

4. Discussion

The finding that the *RBCS3A* promoter is released from correct regulation when transiently transformed on plasmid DNA indicates that it might require integration into the plant chromatin in order to be properly expressed. A second example from plants, for which the requirement for chromatin integration for correct regulation has been shown, is the β-phaseolin gene from bean [4]. The β-phaseolin promoter is strictly embryo-specific when stably transformed into tobacco fused to *GUS* as a reporter gene. In contrast, the same construct gives rise to high-level GUS expression in vegetative tissue after transient transformation, using both particle bombardment and transformation of protoplasts [4]. Neither in the case of the β-phaseolin promoter nor in this study can it be excluded that the discrepancy between transient and stable transformation is due to high copy numbers of the promoter after transient transformation, titrating out a potential repressor complex. However, it is interesting that van der Geest et al. [5] also found a strong MAR located in the β-phaseolin promoter as well as a second MAR in the 3′ flanking region of the gene. While the presence of MARs alone clearly does not prove the involvement of matrix attachment in the observed gene expression phenomena, these correlations increase the awareness about the potential influence of nuclear architecture on gene expression.

So far, no clear evidence for the involvement of MAR-matrix interactions in organ-specific gene expression has been found. One indication comes from the identification of an animal MAR-binding protein which itself is strictly organ-specific [18]. The protein SATB1 is specifically expressed in both mouse and human thymus, but no specific function of the protein in this tissue has been established [18]. In addition, it has been shown that the attachment of the avian β-globin enhancer to the nuclear matrix follows a tissue-specific pattern [19]. Clearly, further research will be necessary to elucidate the function of organ-specific chromatin-matrix interactions and their potential role in gene regulation. The identification of matrix attachment activity of the three *RBCS* promoters will now allow us to compare their binding among nuclear matrix

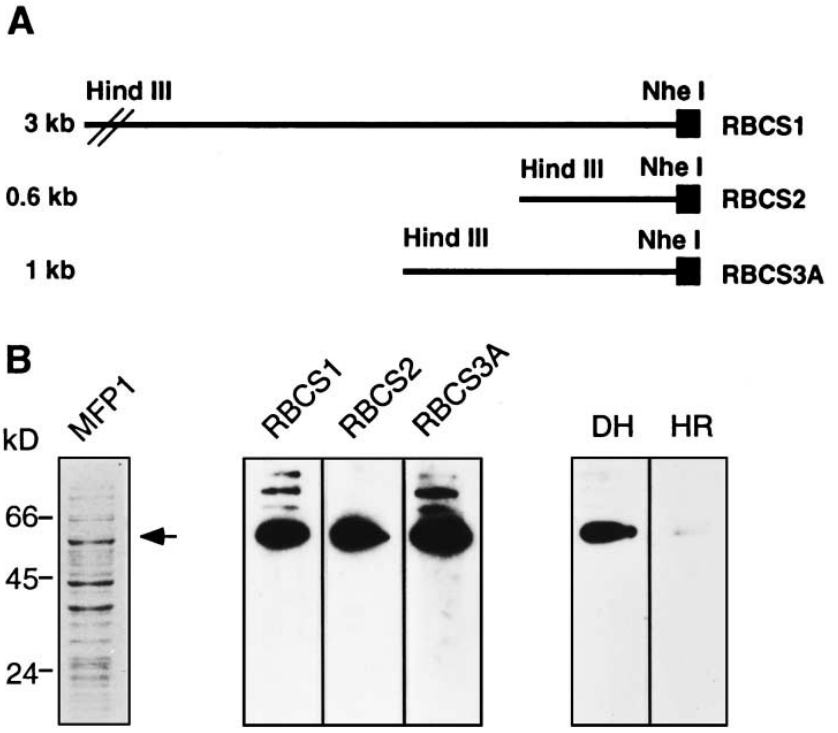

Fig. 3. The plant MAR binding protein MFP1 binds to the three *RBCS* promoter fragments. A: Schematic representation of the three *RBCS* promoter fragments used in this analysis. For *RBCS1* and *RBCS3A* the *Hin*dIII sites correspond to the upstream *Hin*dIII sites of the genomic clones [1]. For *RBCS2* the upstream sequence extends to the first *Sau*3A site at approximately −600 bp, and the *Hin*dIII site is part of the pUC19 polylinker. The *Nhe*I sites are in the *RBCS* coding region, 21 amino acids downstream of the start ATG. B: The left panel shows a protein profile of *E. coli* BL21 cells expressing a histidine-tagged, truncated version of MFP1 (amino acids 295–697, [9]) separated on a 12% SDS-polyacrylamide gel. The arrow indicates the position of the fusion protein. Sizes of the molecular weight markers are indicated on the left. The central and right panel show 'South-western' blot experiments. RBCS1, RBCS2, RBCS3A, *Hin*dIII-*Nhe*I *RBCS* promoter fragments shown in (A); DH, 0.66 kbp *Drosophila* histone H1/H3 spacer MAR DNA [15]; HR, 1.1 kbp AT-rich non-MAR DNA fragment from the repetitive sequence of the *Drosophila* Y chromosome [15].

preparations from different tomato organs, to investigate whether there is a correlation between nuclear matrix binding and promoter activity.

Chromatin-matrix interactions are just one aspect potentially involved in integration-dependent gene regulation. Positioning of nucleosomes and spreading of heterochromatic regions are other aspects which have been shown to influence animal gene expression [6,7,20] and which are likely to operate in plants too. Thus, several scenarios are possible to explain the requirement of chromatin for the silencing of the *RBCS3A* promoter in fruit. Nucleosomal positioning in the *RBCS3A* promoter might differ in leaves and fruits, blocking an enhancer region in fruits that is accessible on naked DNA. Co-activators in yeast and mammals have recently been found to possess histone acetyltransferase activity [17], thus coupling the binding of transcription factors with the acetylation of histones, leading to the loosening of nucleosomes around the transcription start site. If similar mechanisms act on plant promoters too, specific transcription factors involved in opening chromatin structure might only act on the *RBCS1* and *RBCS2* promoters in fruit. In the absence of nucleosomes, additional transcription factors binding to all three promoters could then be responsible for their activity as naked DNA. It would be interesting to attempt to reconstitute nucleosomes in vitro on the *RBCS* promoters [21] and analyze the activity of these promoters in the different organs in transient transformation assays. The accumulating evidence for mechanisms of plant gene regulation other than *cis*-element/*trans*-factor interactions emphasizes the need to investigate these aspects of gene expression and to improve our knowledge about gene regulation at the chromatin level in plants.

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