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Isolation and characterization of the organ-specific and light-inducible promoter of the gene encoding rubisco activase in potato (*Solanum tuberosum*)

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ABSTRACT. Constitutive promoters have been widely used in crop biotechnology applications. Tissue-specific or inducible promoters, however, have advantages in some cases. We isolated the 731-bp 5' flanking sequence of a potato (*Solanum tuberosum*) gene, encoding ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) activase (*RCA*), which was isolated by genome walking. By using GUS as a reporter and with Northern blot analysis, the 702-bp fragment (referred to as *StRCA*p), ranging from nt -731 to -30 relative to the initiation code of the *RCA* gene, was analyzed in transgenic tobacco plants. The activity of *StRCA*p in leaves was 0.4-fold less than that of cauliflower mosaic virus 35S promoter, and was expressed throughout the green part of the light-grown transgenic T_1 seedlings, including cytoledons, leaves and young stems, but not roots. Further deletion analysis revealed that a shorter fragment (nt -249 to -30, *StRCA*p2) retained light-inducible features in cytoledons and leaves, but showed

no detectable activity in young stems and roots. Although the activity of *StRCA*p2 in leaves was reduced significantly compared with that of *StRCA*p, the overall data indicated that *cis*-elements sufficient to regulate organ-specific and light-inducible transcription are within the 220-bp fragment. There is potential for application of *StRCA*p in plant genetic engineering.

Key words: *Solanum tuberosum*; Organ-specific and light-inducible; Promoter; Rubisco activase

INTRODUCTION

Rubisco activase (RCA) belongs to the AAA+ family of ATPases associated with diverse cellular activities (Portis Jr., 2003), and is of a chloroplast-localized enzyme that catalyzes the activation of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). To date, a large number of *RCA* genes have been isolated from over 10 species, including monocotyledon [barley (Rundle and Zielinski, 1991); rice (To et al., 1999); wheat (Law and Crafts-Brandner, 2001); maize (Ayala-Ochoa et al., 2004)], dicotyledon [spinach (Werneke et al., 1988); *Arabidopsis thaliana* (Pilgrim and McClung, 1993); apple (Watillon et al., 1993); tobacco (Qian and Rodermel, 1993); cotton (Salvucci et al., 2003); soybean (Yin et al., 2010); sweet potato (Xu et al., 2010)] as well as *Chlamydomonas reinhardtii* (Roesler and Ogren, 1990), and the functional model of RCA protein in photosynthesis has been well established especially in recent years (Portis Jr., 2003; Salvucci and Crafts-Brandner, 2004; Salvucci et al., 2006; Salvucci, 2008; Portis Jr. et al., 2008).

In most plant species studied, RCA presents α- and β-isoforms. The two forms differ only at the C-terminus (Salvucci et al., 1987; Portis Jr., 2003; Portis Jr. et al., 2008) and arise from the same gene by alternative splicing (Werneke et al., 1988; Pilgrim and McClung, 1993; To et al., 1999), or are produced from related genes (Salvucci et al., 2003). Alternatively, some plants, such as maize, tobacco, bean, and cucumber, only express the β-isoform of RCA (Portis Jr., 2003). Nonetheless, the data collectively demonstrate that the expression of *RCA* transcripts is only in the green part of higher plants and light regulated (Rundle and Zielinski, 1991; Orozco and Ogren, 1993; Watillon et al., 1993; Liu et al., 1996; Xu et al., 2010), indicating that the *RCA* promoters are organ-specific as well as light-inducible.

In contrast to the *RCA* gene and its encoding protein, which has been well studied in a few species, the *RCA* promoters have so far only been isolated and functionally analyzed in spinach (Orozco and Ogren, 1993) and *Arabidopsis* (Liu et al., 1996). Consistent with the transcriptional pattern of the *RCA* gene, both promoters are only active in the green part of plants and light-inducible. Deletion mapping showed that the *cis*-elements sufficient to confer organ-specific and light-regulated transcription in both *RCA* promoters are localized proximal to the transcription start site (Orozco and Ogren, 1993; Liu et al., 1996).

So far, constitutive promoters, in particular the cauliflower mosaic virus (CaMV) 35S promoter, are widely used in plant genetic engineering. It has been accepted, however, that constitutive expression of foreign proteins may impose extra metabolic burden as well as other adverse effects on transgenic plants, finally resulting in abnormal development

(Shelton et al., 2002). In this regard, the organ-specific and light-regulated promoters, such as the *RCA* promoter, have great potential for use in crop biotechnology applications.

Potato (*Solanum tuberosum* L.) is one of the most important crops in the world and ranks fourth after rice, wheat and maize in production. As a kind of heliophyte species, the growth of potato depends greatly on light. The potato *RCA* gene, as well as its promoter sequence, however, remain to be determined. In this study, we isolated the 731-bp 5' flanking sequence of the *RCA* open reading frame (ORF) from potato, and demonstrated that this fragment had promoter activity with organ-specific and light-inducible features. Furthermore, we determined that a 220-bp short sequence adjacent to the site of transcription initiation was sufficient to confer transcriptional response to both organ-specific cues and light.

MATERIAL AND METHODS

Isolation of the *RCA* promoter in potato

Total RNA was firstly extracted from the leaves of a cultivated potato species, *Atlantic* (Chomczynski and Sacchi, 1987), then reverse transcription-polymerase chain reaction (RT-PCR) was performed to amplify the internal part of the *RCA* gene with a pair of degenerate primers (5'-TTCCHCTYATYYTKGGTRTHTGGG-3' and 5'-TCACYTCRTCRTCRTANACHCKG GC-3'), resulting in a 603-bp PCR product. According to the nucleotide sequence of the partial *RCA* gene, three corresponding primers were designed (5' phosphate-labeled primer: 5'-P-CACTTCATCGTCA-3'; the first pair of primers: 5'-GTTCTACTGGGCACCAACTAGG-3' and 5'-GGTTGATTCCCATCTTTCTGAAG-3'; the nested pair of primers: 5'-GATTTCTTCGGT GCTTTGAGGG-3' and 5'-CATACACCCAAGATGAGTGGAA-3'), and 5' RACE was further carried out to identify the 5' end of the *RCA* transcript by following the manufacturer instruction (5'-Full RACE Core Set, TaKaRa, Japan).

Genome walking was performed subsequently to isolate the putative promoter region of the *RCA* gene in potato accordingly (LA PCR™ *in vitro* Cloning Kit, TaKaRa). The genomic DNA from potato *Atlantic* was digested with *Sau*3AI, and ligated with adaptors to result in the template used subsequently. A nested-PCR was then performed to amplify the 5' flanking sequence of the *RCA* gene with two pairs of primers (the first pair of primers: 5'-GTACATATT GTCGTTAGAACGCGTAATACGACTCA-3' and 5'-CGTTAGAACGCGTAATACGACT CACTATAGGGAGA-3'; the nested pair of primers: 5'-GAAGTTCTTGGTGATGTGAA CAGCAAGTTTG-3' and 5'-GGAAAAGACTGTCAACCATACCCTTACCCCT-3') designed according to the identified internal part of the *RCA* gene as well as 5' end sequence of the *RCA* transcript. After gel purification, the resulting PCR products were inserted into T-vectors and transformed into *Escherichia coli* DH5α (Gibco-BRL). The 10 positive clones were subjected to DNA sequencing. Finally, the resulting sequences including the 5' part of the *RCA* gene and the putative promoter region were assembled into one contiguous sequence, which was deposited in Genbank accession No. HQ259068.

Construction of plant expression vectors

To avoid the restriction digestion site of *Hin*dIII at nt -26 (the adenine in initiation

code ATG is assumed to be nt +1), the fragment ranging from nt -731 to -30 (referred to as *StRCA*p hereafter) of the putative *RCA* promoter region was subjected to PCR amplification by using a primer pair (5'-CGCAAGCTTAAATCTAACCTCATA-3' and 5'-ACGTCTAGAATTCTTGATTTGCCTTA-3', the *Hin*dIII and *Bam*HI sites were underlined). The resulting PCR products were purified and double digested with *Hin*dIII and *Bam*HI, then inserted into the *Hin*dIII/*Bam*HI-digested pBI121 (Clontech) to generate pRCAp (Figure 1A), in which the transcription of the *uid*A gene encoding GUS reporter was driven by *StRCA*p, rather than the original CaMV 35S promoter. With the same strategy, two truncated versions of *StRCA*p, which range from nt -498 to -30, and nt -249 to -30, were introduced into pBI121 to result in pRCAp1 and pRCAp2 carrying the expression cassette of *StRCA*p1-*uid*A or *StRCA*p2-*uid*A, respectively (Figure 1A), for identification of the shorter *RCA* promoter fragment sufficient to confer the organ-specific and light-inducible activity.

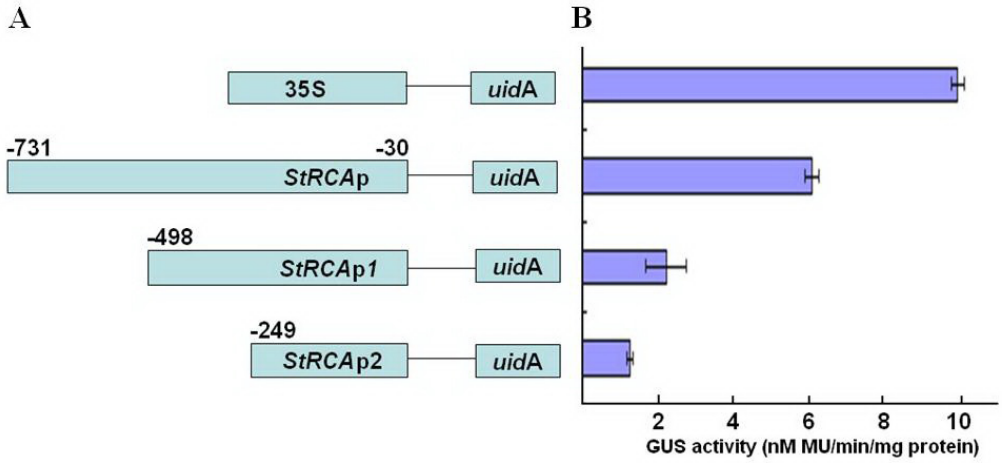

Figure 1. A. Schematic diagrams of the expression cassettes of *StRCA*p-*uid*A, *StRCA*p1-*uid*A and *StRCA*p2-*uid*A, which were generated by fusion of the 5'-flanking regions (from nt -731 to -30, -498 to -30, and -249 to -30) of the potato *RCA* gene with the *uid*A gene. **B.** Horizontal bars represent the average GUS activities in fully expanded leaves, which were collected from different groups of transgenic T_1 tobacco plants at 5- or 6-leaf stage. Each group included at least 10 individual plants. The average GUS activity driven by CaMV 35 S promoter was measured as a control.

Generation of transgenic tobacco plants

The three plant expression vectors as well as pBI121 were introduced into the *Agrobacterium tumefaciens* strain LBA4404 by freeze-thaw. The transgenic plants were generated by *Agrobacterium*-mediated transformation on leaf disks of *Nicotiana tabacum* cv. NC89 as described (Anonymous, 1985). The regenerated kanamycin-resistant plants (T_0) confirmed by PCR and GUS histochemical staining (Jefferson et al., 1987) were grown in a greenhouse, and allowed to generate seeds via self-pollination.

Light treatment, Northern blot analysis and GUS activity assay

Self-pollinated seeds were planted on selection plates containing Murashige and Skoog salts (Murashige and Skoog, 1962), and 200 mg/L kanamycin at 25°C. When the first or second leaf developed, the resistant seedlings were halved and treated with continuous white light and dark, respectively. After 7 days, 10 seedlings were randomly selected from each treatment and subjected to histochemical GUS assay as described by Jefferson et al. (1987). The other etiolated seedlings in the dark were exposed to a light intensity of 2000 lx, and the second leaves were collected from individual seedlings at 0-, 8-, 16-, and 24-h intervals, respectively, to extract total RNA for Northern blot analysis with the labeled *uid*A-specific probe (Xie et al., 2003); while the other light-grown seedlings were transferred to soil and cultured in a green house, the fully expanded leaves were sampled at 5- or 6-leaf stage for quantitative GUS assay (Jefferson et al., 1987).

Computer analysis of the isolated *RCA* promoter sequence

The nucleotide sequence of the isolated *RCA* promoter was aligned by using the BLAST algorithm (Altschul et al., 1997). The putative *cis*-acting-regulatory elements within that sequence were searched against both PlantCARE and PLACE Databases (Prestridge, 1991; Higo et al., 1999; Lescot et al., 2002).

RESULTS

Isolation and sequence analysis of the *RCA* promoter in potato

To isolate the *RCA* promoter in potato, we firstly cloned the 5' end of the *RCA* transcript with the length of 1146 bp (Genbank accession No. HQ259068). The experiment of 5' RACE determined that one adenine at nt -68 relative to the initiation code was the transcription start site, indicating that the 5' untranslation region of *RCA* was 68 bp in length (Figure 2). Based on the identified partial sequence of the *RCA*, a 731-bp 5' flanking sequence of the potato *RCA* gene was isolated by using genomic walking (Figure 2; Genbank accession No. HQ259068). When using BLAST to search the nucleotide database in Genbank, several sequences (for instance, AC151803.1 and AC231666.1) from plants of genus *Solanum* were found to share some similarity with the 731-bp sequence, but without function annotation. A 'TATA' box motif 'TTATTT' at nt -62 and a 'CAAT' box at nt -117 were predicted by searching against the databases of PlantCARE and PLACE (Figure 2). Meanwhile, numbers of conserved light-responsive motifs, such as 'I' box (Terzaghi and Cashmore, 1995) and 'GATA' box (Gilmartin et al., 1990), were identified within the 731-bp fragment (data not shown), suggesting the possible role of this fragment.

Transcriptional activity of *RCA* promoter is organ-specific and light-inducible

To verify the transcriptional activity of the isolated fragment, the transgenic tobacco NC89 plants were transformed with *A. tumefaciens*, carrying pRCAp. Histochemical staining revealed the strong GUS activity in transgenic T_0 tobacco leaves (data not shown), providing

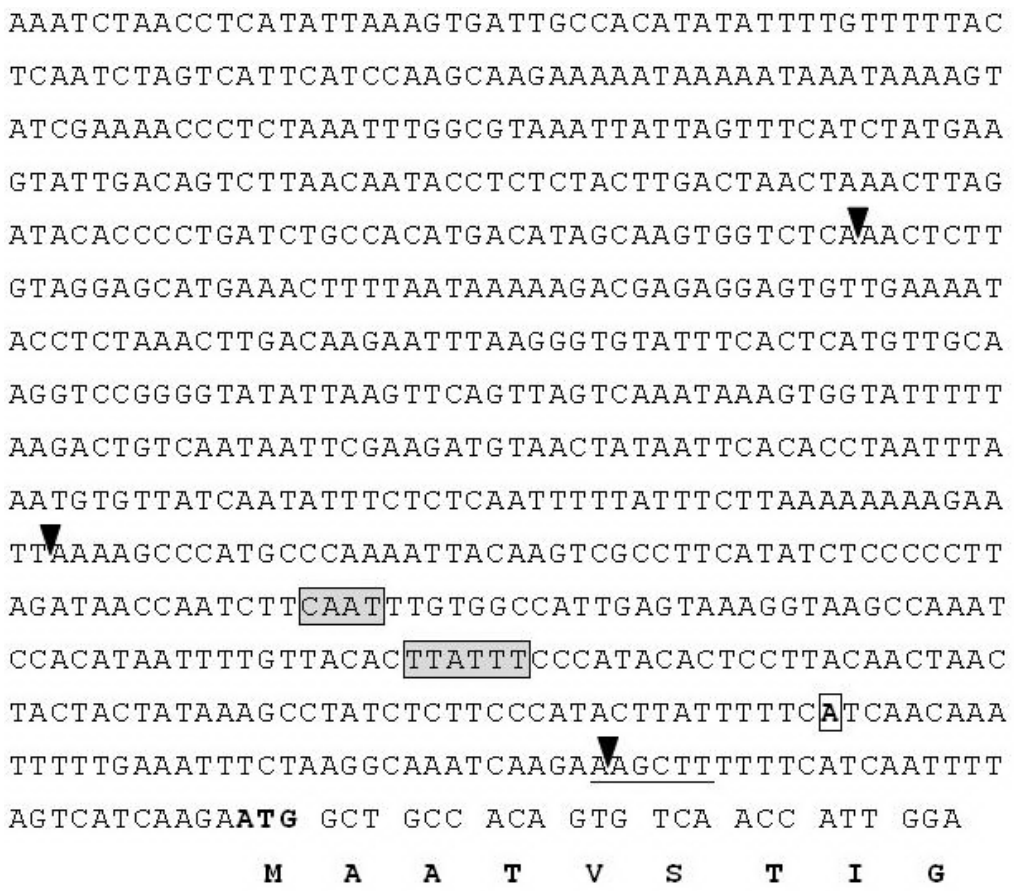

Figure 2. The nucleotide sequence of the 5'-flanking regions (from nt -731 to +27) of the potato *RCA* gene determined in this study. Putative *cis*-acting elements including 'TATA' box and 'CAAT' box were masked with gray. The putative translation start site, ATG, was indicated in bold, and one boxed adenine at nt -68 was identified as the transcription initiation site. The black filled triangles indicate the positions of nt -498, -249 and -30, respectively. The unique restriction site of *Hin*dIII within this sequence is underlined.

evidence that *StRCA*p, the fragment ranging from nt -731 to -30, was able to drive the expression of *uid*A gene in tobacco.

To better evaluate the promoter activity of *StRCA*p, self-pollinated seeds from the transgenic T_0 plants were germinated, and the resulting kanamycin-resistant T_1 seedlings were used in the following experiments. Since the *RCA* promoters in both *Arabidopsis* and spinach are organ-specific and light-inducible (Orozco and Ogren, 1993; Liu et al, 1996), the transcriptional pattern of the *StRCA*p was investigated by using the light- and dark-grown seedlings. Histochemical staining showed that, in contrast to the constitutive GUS expression driven by CaMV 35S promoter (Figure 3A), *StRCA*p failed to drive the GUS expression in dark-grown transgenic seedlings (data not shown but as in Figure 3B), whereas it was active throughout the whole green part of light-grown ones including cotyledons, leaves and young stems, but not roots (Figure 3C). As expected,

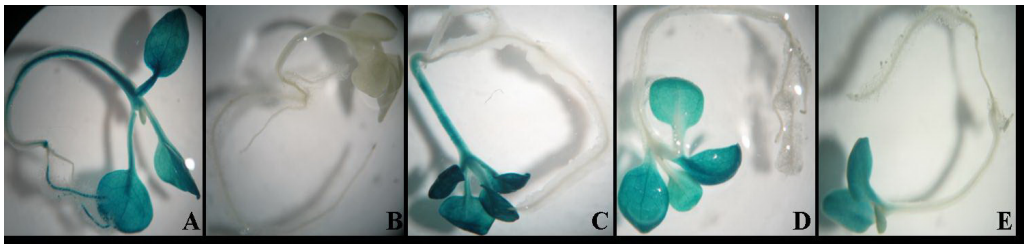

Figure 3. Histochemical localization of GUS expression in transgenic T_1 tobacco seedlings carrying the expression cassette of *RCA*-*uid*A. **A.** CaMV 35S promoter drives GUS expression throughout the whole seedling. **B.** GUS activity was absent in wild-type tobacco seedlings whether under continuous light or in dark for 7 days. Similar results have been observed from the seedlings carrying *StRCA*p-*uid*A, *StRCA*p1-*uid*A or *StRCA*p2-*uid*A, which showed no GUS expression when grown in dark (data not shown). **C. D.** and **E.** The light-grown transgenic seedlings carrying *StRCA*p-*uid*A, *StRCA*p1-*uid*A and *StRCA*p2-*uid*A, respectively, showed significant GUS activity. Notably, the *StRCA*p drove *uid*A expression in cytoledons, leaves and stems, whereas both *StRCA*p1 and *StRCA*p2 directed *uid*A expression in cytoledons and leaves, but not stems.

no GUS activity was detected in either light- or dark-grown tobacco NC89 seedlings (Figure 3B).

The subsequent Northern blot analysis confirmed the light-inducible feature of *StRCA*p. Consistent with the results of histochemical staining, no detectable *uid*A transcripts were accumulated in dark-grown seedlings carrying the *StRCA*p-*uid*A fusion (Figure 4A, lane 1). However, when the dark-grown seedlings were transferred to a light intensity of 2000 lx, the accumulation of *uid*A transcripts could be detected at a 8-h exposure, and increased progressively following the increasing exposure times (Figure 4A, lanes 2-4).

Considering the organ-specific transcriptional feature of *StRCA*p, quantitative GUS activity analysis was performed only with the fully expanded leaves (without the main vein) of the transgenic T_1 plants, which were cultured under continuous white light to 5- or 6-leaf stage. The results showed that the mean GUS activity driven by the *StRCA*p was 0.4-fold less than that of the CaMV 35S promoter (Figure 1B).

A 220-bp fragment of *RCA* promoter sufficient for organ-specific and light-inducible expression

The *cis*-element(s) conferring organ-specific and light-inducible activity was mapped by progressive deletion from the 5' end of *StRCA*p. To retain the putative 'CAAT' box (nt -185) and 'TATA' box (nt -130), two constructs harboring the smaller *StRCA*p fragments ranging from nt -498 to -31 (*StRCA*p1), and nt -289 to -31 (*StRCA*p2), respectively, were produced and used to generate the transgenic tobacco lines carrying *StRCA*p1-*uid*A and *StRCA*p2-*uid*A, respectively. Histochemical staining analysis revealed that no GUS activity was detected in either kind of dark-grown transgenic T_1 seedling (data not shown but as in Figure 3B), whereas GUS activity was detected in light-grown ones of cotyledons and leaves, instead of young stems and roots (Figure 3D and E).

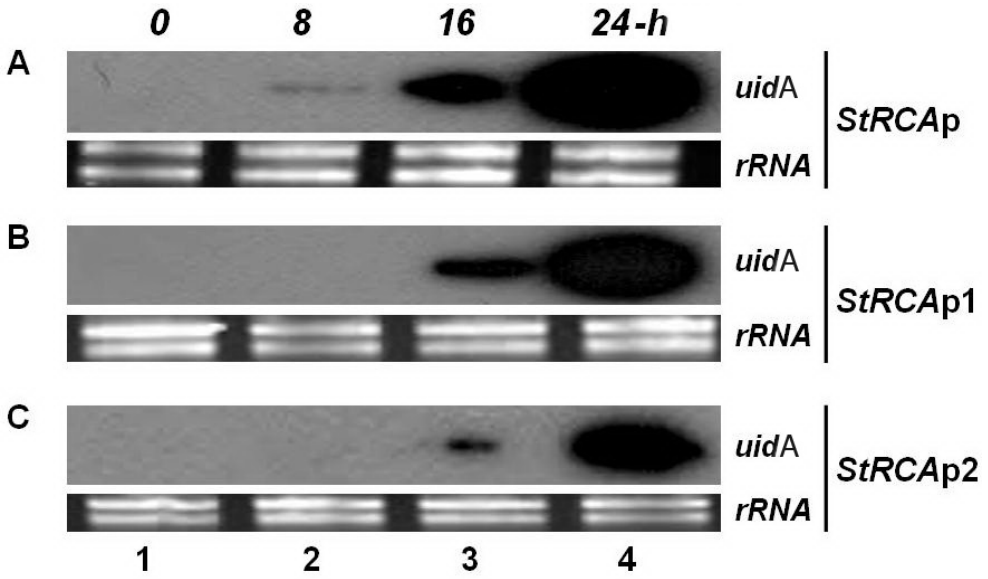

Figure 4. Accumulation of *uid*A mRNA in transgenic T_1 tobacco seedlings carrying **A.** *StRCA*p-*uid*A, **B.** S*tRCA*p1-*uid*A or **C.** *StRCA*p2-*uid*A. The seedlings were first etiolated in the dark for 7 days, then exposed to a light intensity of 2000 lx for 0-, 8-, 16-, and 24-h intervals, respectively. Total RNA extracted from the second leaves was loaded for Northern blot analysis by using a *uid*A-specific probe. Bands corresponding to the transcripts of *uid*A are indicated. The rRNAs are shown to indicate the relative equivalency of the samples loaded.

The light-inducible expression of both *StRCA*p1 and *StRCA*p2 was further verified by Northern blot analysis. The *uid*A transcripts were under the detectable level in dark-grown seedlings carrying either *StRCA*p1-*uid*A or *StRCA*p2-*uid*A (Figure 4B and C, lane 1). However, when the seedlings were transferred to a light intensity of 2000 lx, the expression of *uid*A transcripts was increased stepwise following the prolonged exposure times (Figure 4B and C, lanes 2-4), like the behavior of *StRCA*p under the light treatment (Figure 4A). It was noteworthy, however, that the accumulation of *uid*A was not detected until after 16 h of continuous exposure when driven by either *StRCA*p1 or *StRCA*p2, suggesting that removal of the 5' end of *StRCA*p greatly affected its transcriptional activity.

The reduced activities of *StRCA*p1 and *StRCA*p2 in leaves were confirmed by quantitative GUS activity analysis. The results showed the two truncated fragments retained the promoter activities, which, however, were only ~37 and ~23% compared with that of *StRCA*p. Nonetheless, the collective data established that the fragment extracted from nt -249 to -30 (*StRCA*p2) was sufficient to drive GUS expression in light-inducible and organ-specific transcriptional manner.

DISCUSSION

In this study, a 731-bp fragment upstream of the initiation code of the *RCA* gene in potato was isolated and analyzed. GUS activity assay and Northern blot analysis performed with transgenic tobacco plants revealed that *StRCA*p, ranging from nt -731 to -30, was able

to drive the *uid*A gene expression in light but not darkness. Additionally, the GUS activity was not detectable in roots, but throughout the cytoledons, leaves and young stems, the whole green part of tobacco seedling. Taken together, these data clearly support the *StRCA*p from potato as a promoter with light-inducible and organ-specific features, resembling the behavior of *RCA* promoters in *Arabidopsis* and spinach reported previously (Orozco and Ogren, 1993; Liu et al., 1996).

It has been established that, for the *RCA* promoter from either *Arabidopsis* or spinach, a ~300-bp fragment adjacent to the transcription start site is sufficient to confer light-inducible and organ-specific transcription (Orozco and Ogren, 1993; Liu et al., 1996). A similar result was obtained from the potato *RCA* promoter, the *cis*-elements, which are necessary for light-inducible and organ-specific transcription also localized within a 220-bp fragment just upstream of the transcription start site, as evidence that two 5' progressively truncated versions, *StRCA*p1 and *StRCA*p2, retained light-inducible and organ-specific manner in transgenic tobacco plant.

GUS activity analysis showed that the transcriptional activities of two truncated versions, *StRCA*p1 and *StRCA*p2, in leaves were reduced significantly compared with that of *StRCA*p, suggesting that the deleted 483-bp fragment might harbor *cis*-regulatory element(s) such as transcriptional enhancer(s) (Halfon, 2006). Except for the reduced promoter activity, both *StRCA*p1 and *StRCA*p2 were only active in cytoledons and leaves, unlike the full length of *StRCA*p, which drove the *uid*A expression in not only cytoledons and leaves, but also young stems. It has been determined that the *RCA* transcripts in stem are much less abundant than those in leaves (Liu et al., 1996), that is, the activity of a mutated *RCA* promoter might be completely shut down in stems but only reduced in leaves. In this case, therefore, it is conceivable that the failure of *StRCA*p1 and *StRCA*p2 to drive GUS expression in young stems might be correlated with their significantly reduced activity in leaves. However, we cannot rule out the possibility that the removed fragment from -731 to -249 might contain some yet-unknown *cis*-element(s) essential for regulation of *StRCA*p activity in young stems.

The light-inducible and organ-specific features of *StRCA*p present specific advantages in plant genetic engineering for leaf-consuming pests or foliar disease resistance. For instance, in potato genetic engineering, *StRCA*p could be employed to express defensive genes only in the green part of plant to specifically resist Colorado beetle (Weber, 2003), powdery mildew (Park and Jones, 2008) or aphid, which transmit several important potato viruses (Hodgson, 1991). The application of *StRCA*p would not only reduce the metabolic burden imposed on transgenic potato plants, but also reduce concern over food safety (Stewart et al., 2000; Shelton et al., 2002) since the edible part of potato is a tuber that grows underground. Additionally, considering cisgenesis, a scientific theory proposed recently with regard to the genetically modified plants (Jacobsen and Schouten, 2007; Schouten and Jacobsen, 2008), extra values would be expected when the *StRCA*p from a potato species was applied back to potato genetic engineering.

In summary, the isolated 702-bp fragment (*StRCA*p) upstream of the *RCA* ORF in potato showed inducible and organ-specific transcriptional activity, which, however, could be conferred by only the 220-bp short fragment proximal to the 3' end of *StRCA*p. So far, several light-inducible and organ-specific promoters have been determined (Ueda et al., 1989; Orozco and Ogren, 1993; Liu et al., 1996; Nomura et al., 2000; de Souza et al., 2009), while the characterization of *StRCA*p has provided an alternative promoter with similar characteristics for plant

genetic engineering and shed some light on the yet-unknown *RCA* transcriptional patterns in potato. Given the lower activity of *StRCA*p compared with that of CaMV 35S promoter, the ongoing study is focused on improving the activity of this promoter but without disturbing its light-inducible and organ-specific manner.

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