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ORIGINAL ARTICLE

Promoter analysis of the sweet potato ADP-glucose pyrophosphorylase gene *IbAGP1* in *Nicotiana tabacum*

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Abstract

Key message **The *IbAGP1* gene of sweet potato (*Ipomoea batatas*) encodes the sucrose-inducible small subunit of ADP-glucose pyrophosphorylase. Through expression analysis of 5′-truncations and synthetic forms of the *IbAGP1* promoter in transgenic tobacco, we show that SURE-Like elements and W-box elements of the promoter contribute to the sucrose inducibility of this gene.**

Abstract Sweet potato (*Ipomoea batatas*) contains two genes (*IbAGP1* and *IbAGP2*) encoding the catalytically active small subunits of ADP-glucose pyrophosphorylase, an enzyme with an important role in regulating starch synthesis in higher plants. Previous studies have shown that *IbAGP1* is expressed in the storage roots, leaves, and stem tissues of sweet potato, and its transcript is strongly induced by applying sucrose exogenously to detached leaves. To investigate the tissue-specific expression of the *IbAGP1* promoter, a series of 5′-truncated promoters extending from bases −1913, −1598, −1298, −1053, −716, and −286 to base +75 were used to drive the expression of the β-glucuronidase reporter gene (*GUS*) in tobacco plants (*Nicotiana tabacum*). Histochemical and fluorometric GUS assays showed that (1) *GUS* expression driven by the longest fragment (1989 bp) of the *IbAGP1* promoter was detected in vegetative tissues (roots, stems, leaves), (2) fragments extending to −1053 or beyond retained strong *GUS* expression in roots, stems, and leaves, whereas further 5′-deletions resulted in considerable reduction in GUS activity, and (3) the series of 5′-truncated promoters responded differently to exogenously applied sucrose. The 1989-bp *IbAGP1* promoter contains five sequences (two AATAAAA, one AATAAAAAA, and two AATAAATAAA) that are similar to sucrose-responsive elements (SURE). These SURE-Like sequences are found at nucleotide positions −1273, −1239, −681, −610, and −189. Moreover, putative W-box elements are found at positions −1985, −1434, −750, and −578. Synthetic promoters containing tandem repeats of the 4X SURE-Like or 4X W-box upstream from a minimal *CaMV35S* promoter-*GUS* fusion showed significant expression in transgenic tobacco in response to exogenous sucrose. These results show that SURE-Like elements and W-box elements of the *IbAGP1* promoter contribute to the sucrose inducibility of this gene.

Keywords *Ipomoea batatas* · *IbAGP1* · Promoter analysis · *Nicotiana tabacum* · GUS expression

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Introduction

Starch is an important carbohydrate and the primary energy source in plants. Starch biosynthesis occurs mainly by the participation of three enzymes: ADP-glucose

pyrophosphorylase (AGPase), starch synthase, and branching enzymes. The first enzyme in starch biosynthesis in plants is AGPase. It catalyzes the synthesis of ADP-glucose (a glucosyl substrate for the synthesis of starch polymers) from glucose-1-phosphate coupled by the hydrolysis of ATP. Many researchers have revealed that the AGPase catalyzes the rate limiting step in starch biosynthesis in higher plants. ADP-glucose is then used by starch synthase for the synthesis of polyglucans.

AGPase is a heterotetramer in higher plants and is composed of two small and two large subunits. In Arabidopsis, six genes encode AGPase, and two of these genes encode for S subunits (APS1 and APS2) and four encode L subunits (APL1-APL4; Crevillen et al. 2003, 2005; Ventriglia et al. 2008). The large subunits are encoded by members from a multi-gene family and their expressions are organ-specific in higher plants (Chen et al. 1998; La Cognata et al. 1995; Villand et al. 1992, 1993). The small subunits, however, are not consistently found in multi-gene families. Only a single small subunit gene has been found in potato (Nakata et al. 1991), rice (Anderson et al. 1989), and tomato (Li et al. 2002). Two small subunit isoforms in bean (Weber et al. 1995) and maize (Prioul et al. 1994) are organ-specific, while the two small subunit isoforms in sweet potato (Bae and Liu 1997), pea (Burgess et al. 1997), and *Perilla frutescens* (Choi et al. 2001) are non-organ-specific. In higher plants, the catalytic and regulatory properties of AGPase are a product of synergistic interactions between the small subunits and large subunits. The small subunits are mainly responsible for the catalytic activity, and the large subunits execute regulatory roles. In Arabidopsis the only functional small subunit is APS1, while the *APS2* coding gene may be in a process of pseudogenization (Crevillen et al. 2003, 2005). In addition to their regulatory roles, the two of large subunits (APL1 and APL2) from Arabidopsis have catalytic activity, while the other two homologs have lost their catalytic capacity during evolution (Ventriglia et al. 2008).

AGPase genes are transcriptionally regulated by the application of exogenous sucrose to detached leaves. In assays using detached leaves of Arabidopsis, tomato, and potato, some large subunit isoforms showed sucrose-inducible expression, whereas others did not (La Cognata et al. 1995; Li et al. 2002; Muller-Rober et al. 1990; Sokolov et al. 1998). In potato, sAGP, a single small subunit gene was observed to be insensitive to the application of exogenous sucrose to detached leaves (Muller-Rober et al. 1990), while transcripts of the only identified small subunit gene in tomato (AgpB) were strongly elevated following the application of exogenous sucrose to detached leaves and fruits (Li et al. 2002). A single functional Arabidopsis small subunit gene (ApS1) was originally reported to be sucrose-inducible based on results obtained from sucrose-fed detached leaves (Sokolov et al. 1998). However, this same small subunit gene showed a sucrose-insensitive expression pattern under normal physiological conditions (Crevillen et al. 2005) when mature Arabidopsis plants were irrigated with sucrose-containing MS medium. In sweet potato, two small subunit genes *ibAGP-sTL1* and *ibAGP-sTL2* were isolated from tuberous roots and leaves, and the expression of *ibAGP-sTL1* but not *ibAGP-sTL2* is induced by exogenous sucrose (Bae and Liu 1997). Based on an analysis of AGPase expression in different kinds of root tissues and in different developmental stages by light and endogenous sucrose, the sweet potato AGPase gene was shown to be closely associated with the differentiation of thickening pigmented roots (Kim et al. 2002).

Promoters of AGPase genes have been isolated from several plants for characterization. A 3.2-kb promoter of the large subunit gene of the potato AGPase (AGPase S) was active in various starch-containing cells, including guard cells, tuber parenchyma cells, the starch sheath layer of stems, and petioles in transgenic potato and tobacco (Muller-Rober et al. 1994). The promoter of the AGPase small subunit gene (sAGP) of potato conferred high expression in stem, petiole, and tuber in transgenic potato plants, and its spatial expression patterns were regulated by *cis*-elements located both upstream and downstream from its coding region (Nakata and Okita 1996). The promoter of the small subunit AGPase of rice (OsAGP) directed endosperm-specific expression in transgenic maize (Russell and Fromm 1997). The promoter of the small subunit of Arabidopsis AGPase mediated okadaic acid-sensitive uidA expression in starch-synthesizing tissues and cells including leaf mesophyll cells, guard cells, sub-epidermal cortical layers of the stem, root cap columella cells, carpels, and sepals in Arabidopsis (Siedlecka et al. 2003). Sequence analysis of two promoters of the small subunits of sweet potato AGPase, *IbAGP1,* and *IbAGP2* identified putative sucrose-responsive elements on the *IbAGP1* promoter, and conversely, putative sucrose-starvation elements on the *IbAGP2* promoter. Transient expression analyses on transverse storage root sections revealed that the *IbAGP1* and *IbAGP2* promoters directed strong expression in the sweet potato storage roots. In the storage roots, *IbAGP1* promoter activity became stronger with increasing endogenous sucrose levels, while *IbAGP2* promoter activity became markedly weaker (Kwak et al. 2006).

The strong constitutive promoters that have been widely used in dicot transgenic plants are mainly from prokaryotes such as the 35S promoter from cauliflower mosaic virus (CaMV35S) (Odell et al. 1985). In this study, we tested a series of different lengths of 5′ upstream region of the small subunit AGPase gene of sweet potato, *IbAGP1*, in transgenic tobacco (*Nicotiana tabacum*) plants for their activity

of driving the GUS reporter gene. We found the promoter of the sweet potato *IbAGP1* gene had a decent level of constitutive expression (one-third of the 35S promoter's activity) in different vegetative tissues in tobacco, which could be further boosted to a high level in leaves and stems (more than two times of the 35S promoter's activity) by adding exogenous sucrose. We further examined some cis elements in the *IbAGP1* promoter for their function in sucrose inducibility by using a tobacco transient assay. The results showed that the SURE-like element and W-box are *cis* elements in mediating the *IbAGP1* promoter's response to sucrose in detached leaves. The "constitutive and tunable" property of the *IbAGP1* promoter and the identified functional *cis* elements can be useful in certain applications in agro-biotechnology.

Materials and methods

Plant materials and bacterial strains

Sweet potato plants (*Ipomoea batatas* cv. Nanshu 88) and tobacco plants (*N. tabacum* cv. Xanthi) were grown on sterile MS medium before being used for transformation. *Escherichia coli* (strain DH5a), was used for the cloning and propagation of all recombinant plasmid vectors. *Agrobacterium tumefaciens* (strain GV3101) was used for plant transformation. Plasmid pBI121 (Clontech, USA) was used to generate promoter fragment constructs.

Construction of the *IbAGP1* promoter::GUS expression vector and the artificial promoter expression vectors

To construct the chimeric genes consisting of the GUS coding sequence driven by various lengths of the *IbAGP1* promoter, a series of promoter fragments from −1913 to +75 were obtained by PCR amplification using sweet potato Nanshu 88 genomic DNA as a template. The primers with additional restriction sites (a *Hin*dIII site at the 5′ end and a *Bam*HI site at the 3′ end) were designed as shown in Table S1. PCR reaction was carried out in a 25-μL volume for 33 cycles under the following conditions: 94 °C for 5 min; 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1.5 min. After gel-purification, PCR products were sub-cloned into pUC-T vector (Sangon, Shanghai, China). The resulting constructs were digested with *Hin*dIII/*Bam*HI and then cloned into the corresponding sites of binary expression vector pBI121. A total of seven plasmids were generated. After verification by sequencing, all deletion constructs were transformed into *A. tumefaciens* strain GV3101 by electroporation.

The 46-bp core fragment of CaMV 35S promoter was used as a minimal promoter to replace the 2X35S promoter of pBI121 and to control the GUS expression as the background. For examining the function of the two *cis*-acting elements, SURE-Like and W-box of the *IbAGP1* promoter, two pairs of Oligo DNA were annealed, respectively, to make 4X SURE-Like or 4X W-box fragments with *Eco*RI/*Bam*HI overhang (Table S2). The two fragments upstream the 35S minimal promoters were ligated to obtain the expression vectors, respectively (Fig. 5). After verification by sequencing, all artificial promoter constructs were transformed into *A. tumefaciens* strain GV3101 by electroporation.

Plant transformation and identification

Agrobacterium tumefaciens strain GV3101 containing the *IbAGP1* promoter::GUS constructs was allowed to grow on YEB medium (10 g/L Bacto peptone, 1 g/L yeast extract, 5 g/L sucrose, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 100 μg/mL rifampicin and 50 μg/mL kanamycin). A single colony was inoculated into 5 mL of LB broth containing rifampicin (100 μg/mL) and kanamycin (50 μg/mL) at 28 °C for 2 days, and then 1 mL bacterial culture was transferred in 50 mL of fresh LB with rifampicin (100 μg/mL) and kanamycin (50 μg/mL) and co-cultivated at 28 °C overnight. Agrobacterium cells were centrifuged at 5000 g for 10 min, and subsequently re-suspended in infiltration media, containing 0.19 MS salts, 0.19 B5 vitamins, pH 5.8, 1 % (w/v) glucose, 2 % (w/v) sucrose, and adjusted to an OD_{600} of 0.5 for transformation.

Leaf discs of *N. tabacum,* cut into approximately 0.5 cm × 0.5 cm were incubated with *A. tumefaciens* culture for 10 min with slow shaking and then transferred to a sterile filter paper to remove excess bacteria. After 2 days of cultivation at 25 °C in dark, the infected discs were transferred to MS medium containing 8 g/L agar, 30 g/L sucrose, 500 mg/L cefotaxime, 0.1 mg/L NAA, 2.0 mg/L N6-enzyladenine (6-BA), and 100 mg/L kanamycin for selection. The explants were cultivated at 25 °C in a 16/8 h (light/dark) photoperiod and transplanted to fresh MS medium with 500 mg/L cefotaxime and 100 mg/L kanamycin for selection every 2 weeks. Adventitious shoots were excised and transferred to the hormone-free MS medium supplemented with 100 mg/L kanamycin until roots were induced from regenerated stems. The plantlets were transplanted in soil and grown in a greenhouse.

To verify the presence of transgenes, putative transgenic tobacco plants were screened preliminarily by PCR. Total genomic DNA was isolated from all the kanamycin-resistant plants using the modified CTAB extraction method as previously described (Luo et al. 2006). PCR amplification was performed in a reaction volume of 25μL containing 2.5 μL 10 × buffer, 0.5 μL 10 mM dNTPs, 1 μL plant genomic DNA (50 ng/L), 0.5 μL each of forward and

reverse primers (10 μM) and 0.2 μL *Taq* polymerase (5 U/μL). PCR primer pairs were as follows 5′-GCAAGCT TACTGATACTTTTGGTGACTGC-3′ (forward primer), 5′-CTGGATCCGCGC- TACCACTCTGAGCTCCTG-3′ (reverse primer) for amplification of GUS gene fragments. PCR conditions were 94 °C for 5 min; 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, 33 cycles in total. PCR amplification was performed using a thermocycler (Thermo Scientific Arktik TCA0001).

Tobacco transient assays

Tobacco plants were grown in a growth chamber for 1 month after germination. Healthy leaves from the middle part of each plant were used for bacterial infection. GV3101 containing the artificial promoter::GUS constructs were inoculated into 5 mL of LB broth containing rifampicin (100 μg/mL) and kanamycin (50 μg/mL) at 28 °C for 2 days. GV3101 cells were centrifuged at 5000 *g* for 10 min and subsequently re-suspended in 10 ml distilled water, containing 10 mM $MgCl_2$, 100 μM Acetosyringone (AS) and adjusted to an OD_{600} of 0.5 for injection. Suspended GV3101 cells were infiltrated into tobacco leaves from the back side with a 1-ml needleless syringe. Infected tobacco plants were grown in the growth chamber under a 16/8-h (light/dark) photoperiod for 1 week.

Sucrose induction treatment in detached leaves

For the sucrose feeding experiment, T2 homozygous transgenic tobacco plants were grown in soil in the growth chamber under a 16/8-h (light/dark) photoperiod. Mature leaves with petioles, shoots, and roots were excised at the end of the dark period and incubated in distilled water (DW) containing 6 % sucrose, at 25 °C for 16 h under constant light (light intensity 3000 lux). The explants were then collected.

Histochemical and fluorometric GUS assays

Histochemical staining for beta-glucuronidase (GUS) activity was performed according to the method of (Jefferson et al. 1987). Transgenic plants were incubated in the GUS reaction buffer (1mM5-bromo-4-chloro-3-indolyl-b-D-glucuronide, 100 mM phosphate buffer, pH 7.0, 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, and 10 mM EDTA at 37 °C for 18–24 h. For better visualization of the stained tissue, plant tissues were rinsed with an ethanol series for at least 1 h to remove chlorophyll at room temperature and then observed for microscopy. A fluorometric GUS assay was performed according to a previously published method (Zheng et al. 2007). For fluorometric determination of GUS activity, various tissues of transgenic or control plants were homogenized in GUS extraction buffer (50 mM phosphate buffer, pH7.0, 10 mM EDTA, 0.1 % TritonX-100, 0.1 % sodiumlauryl sarcosine, and 10 mM β-mercaptoethanol). The homogenate was then centrifuged for 10 min at 12,000*g* at 4 °C, and the GUS activity of the supernatant was assessed according to the method described by (Jefferson et al. 1987). Aliquots of the extracts (100 μL) were added to 1 mL of assay buffer (extraction buffer containing 1 m MMU), pre-warmed, and incubated at 37 °C After 0, 5, 10, 20, 30, and 60 min of incubation; 100 μL samples were removed and placed in 0.9 ml stop buffer (200 μM Na_2CO_3). Fluorescence was measured using a Multi-Detection Microplate Reader (Bio-TEK Synergy™ HT). Protein concentration of the samples was determined by the procedure of Bradford (Bradford 1976). GUS activity was expressed as picomole 4-MU per minute per milligram protein. Three replicates were performed for each sample. The data were analyzed by Duncan's multiple range tests.

Results

Sequence analysis of the promoter region *IbAGP1*

To investigate the potential transcriptional regulatory elements involved in expression, the full-length *IbAGP1* promoter (from −1913 to +1) was subjected to a computational analysis [http://www.dna.affrc.go.jp/htdocs/PLACE/(Higo et al. 1999); http://bioinformatics.psb.ugent.be/webtools/plantcare/ (Lescot et al. 2002)]. Several *cis*-acting elements responsible for light response, such as Box4, G-box, GAG-motif, and Sp1, were found in the *IbAGP1* promoter (Table S3). Two abscisic acid responsive elements, ABRE (TACGTG) and ABRE (CACGTG), one auxin responsive element, AuxRR-core (GGTCCAT), and two MeJA responsive elements, CGTA-motif and GACG-motif, were also found in *ibAGP1* promoter (Table S3). These light or hormone response elements are probably involved in regulating starch synthesis and tuber development of sweet potato.

As mentioned in the introduction, there was no ideal sucrose-responsive element (SURE), AATAGAAAA or AATACTAAT sequence (Grierson et al. 1994), in the *IbAGP1* promoter (Kwak et al. 2006). However, five potential SURE-Like elements (two AATAAAA, one AATAAAAAA, and two AATAAATAAA) at nucleotide positions −1273, −1239, −681, −610 and −189 were identified in the *IbAGP1* promoter (Fig. 1; Table S3). WRKY proteins bind to the DNA sequence motif (T)TGAC(C/T), known as the W-box (Eulgem et al. 2000; Rushton et al. 2010). A WRKY transcription factor in barley, SUSIBA2, has been shown to bind to W-box and SUREs in the promoter of the *isoamylase1* gene as an

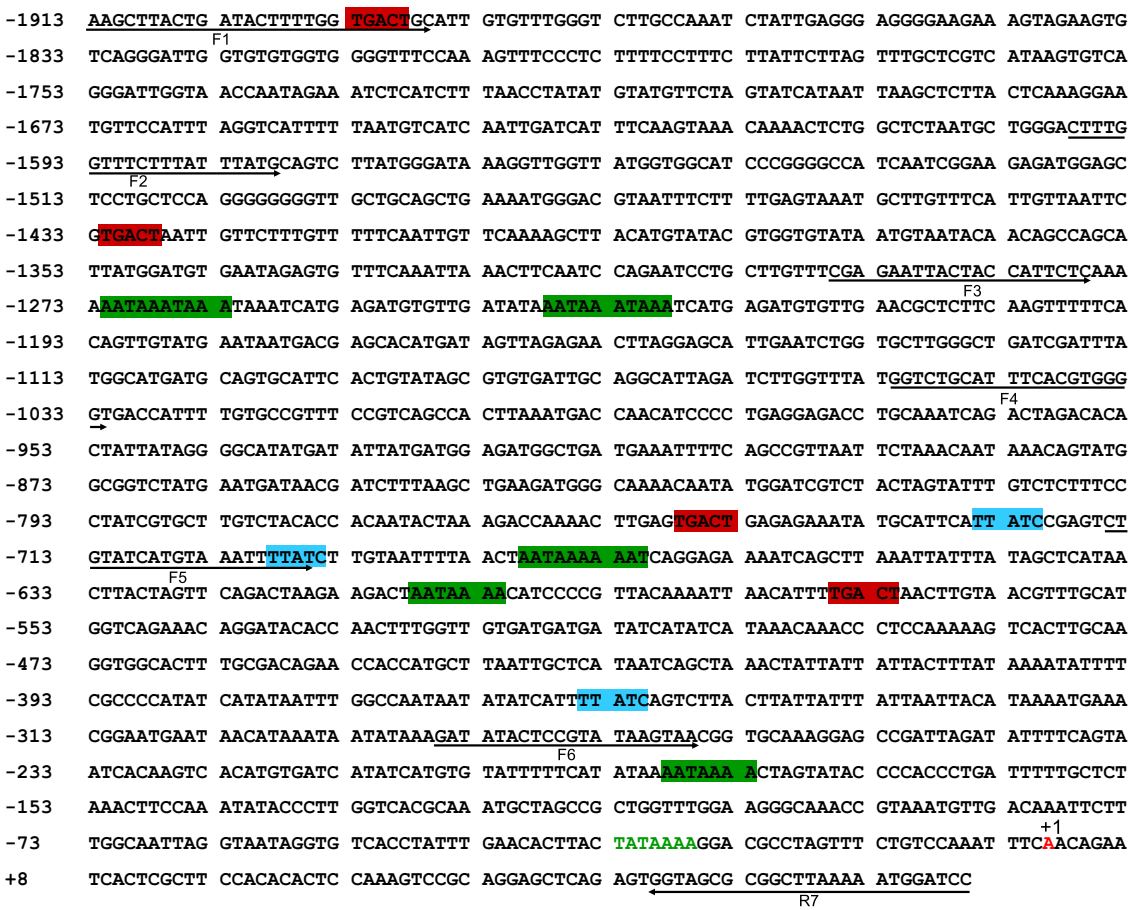

Fig. 1 Nucleotide sequence and annotation of the *IbAGP1*. The 1989-bp upstream of the *IbAGP1* promoter from −1913 to +75 is shown. The putative transcription start site is designated as "+1" and the TATA box is indicated in *green*. The positions of the 5′ upstream of the fusions are indicated by *arrows*, respectively. All putative cis-acting elements are *boxed* in *colors*, including SURE-Like *green square* (sucrose responsive element), W-box *red square* (WRKY protein-binding element) and SRE *sky blue square* (sugar-repressive relative element) (color figure online)

activator (Sun et al. 2003). We identified four W-box sequences (WRKY protein binding element; TGACT) at the −1985, −1434, −750, −578 nucleotide positions of the *IbAGP1* promoter. In Arabidopsis, the promoters of down-regulated genes were enriched for TTATCC motifs that resemble the sugar-repressive element (SRE) (Tatematsu et al. 2005). We also found three SRE boxes located at positions −726, −700, and −356 in the 5′-flanking region of the *IbAGP1* gene. Presumably, these sequences are involved in transcriptional regulation of the *IbAGP1* gene. These *cis* elements in the *IbAGP1* promoter may be involved in sucrose response.

Deletion analysis of the *IbAGP1* promoter in transgenic tobacco

The 5′-flanking region (from −1913 to +75) of *IbAGP1* was isolated from sweet potato Nanshu 88 by PCR amplification. Sequence analysis of the amplified fragment (Fig. 1) revealed 99 % identity with the upstream region of the *IbAGP1* gene reported previously (Noh et al. 2004; Kwak et al. 2006, 2007). To investigate the regulation of the *IbAGP1* promoter, truncated promoters of different lengths (from approximately 0.36 to 1.9 kb) were fused to the GUS reporter gene (Fig. 2). As a result, six promoter constructs were generated, including *IbAGP1*-I (−1913 to +75), *IbAGP1*-II (−1598 to +75), *IbAGP1*-III (−1298 to +75), *IbAGP1*-IV (−1053 to +75), *IbAGP1*-V −716 to +75), and *IbAGP1*-VI (−286 to +75) (Fig. 2). All constructs were introduced into tobacco plants (*N. tabacum* cv. Xanthi) by *A. tumefaciens* mediated transformation. More than 25 putative transgenic plants were obtained for each construct. The stable integration of T-DNA into the genome of the transgenic plants was verified by PCR analysis, and 31 out of 37 (approximately 84 %) transformants contained the *GUS* transgene (Fig. S1).

Expression of the *GUS* gene under the control of different lengths of the *IbAGP1* promoter was analyzed in

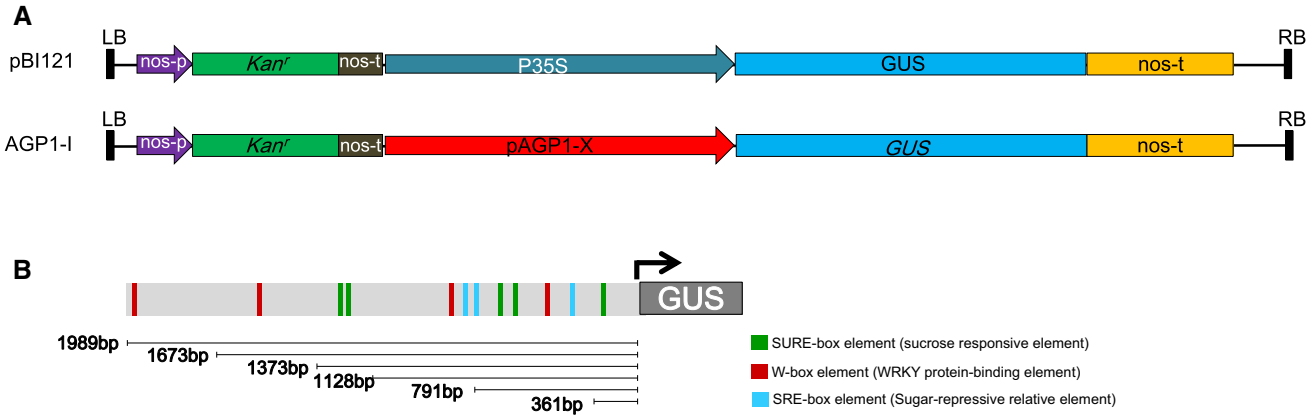

Fig. 2 Deletion analysis of the *IbAGP1* promoter. **a** Schematic representations of the T-DNA regions of ibAGP1 express vectors. *RB* right border, *LB* left border; *Kan*^{r} kanamycin selection marker, *35S* CaMV 35S promoter, *GUS* β-glucuronidase gene, *nos-t* the terminator of the nopaline synthase gene, *pAGP1* the promoter of the *IbAGP1* gene. X represents different *IbAGP1* promoter deletions (I, II, III, IV, V and VI). **b** A series of promoter truncations (from 361 bp to 1989 bp). Different potential transcription binding DNA motifs are illustrated

vegetative tissues of 4-week-old transgenic tobacco plants. GUS histochemical assays were performed in various organs of transgenic tobacco plants harboring the different lengths of the *IbAGP1* promoter. In agreement with previous reports (Kwak et al. 2006), GUS histochemical assays showed that GUS expression driven by the full-length *IbAGP1* promoter was detected in mature leaves and roots (Fig. 3). We found that GUS expression driven by the full-length *IbAGP1* promoter was also detected in stems. Among all three tissues (leaves, stems and roots) examined, the full-length *IbAGP1* promoter exhibited an activity which was about one-third of the 35S promoter (Fig. 4 and Table S4). However, upon sucrose induction, the activity of the *IbAGP1* promoter was significantly increased, more than two times of the 35S promoter's in both above-ground tissues (leaves and stems). The induction of the *IbAGP1* promoter by sucrose was also observed in roots, but at a lower level than in stems and leaves (Fig. 4).

Deletion analysis of the *IbAGP1* promoter region revealed a series of 5′ deletions of the promoters gradually compromised its constitutive activity as reflected by the GUS activity (Figs. 3, 4 and Table S4). In *IbAGP1*-II (−1598 to +75) promoter, sucrose-induced GUS activity was dramatically dropped compared with that of *IbAGP1*-I (−1913 to +75) promoter. There were several common enhancer regions in the fragment from −1913 to −1598, such as CAAT-boxes (CAATT at −1624 and CAAT at −1740 and −1643) (Table S3), which might be required for the induction of *IbAGP1*-I promoter by sucrose. For *IbAGP1*-III and *IbAGP1*-IV, GUS expression levels were comparable without sugar treatment (Fig. 4). However, upon sucrose treatment, GUS expression was significantly reduced with *IbAGP1*-IV than with *IbAGP1*-III in leaves and stems, but not in roots (Fig. 4). It suggested that the two pairs of SURE-Like elements in −1273 and −1239 may play a specific role on sucrose inducibility in leaves and stems. There was a similar change between *IbAGP1*-V and *IbAGP1*-VI, which could be explained by the two pairs of SURE-like elements at −681 and −610.

There are three SRE boxes located at positions −726, −700, and −356 of the *IbAGP1* promoter (Fig. 1 and Table S3). Comparison among *IbAGP1*-IV (−1053 to +75), *IbAGP1*-V (−716 to +75), and *IbAGP1*-VI (−286 to +75) suggested that the absence of SRE elements did not influence the sucrose induction of GUS. There are four W-box elements found in the *IbAGP1* promoter, which are located in −1985 (in *IbAGP1*-I), −1434 (in *IbAGP1*-II), −750 (in *IbAGP1*-III and *IbAGP1*-IV), and −578 (in *IbAGP1*-V). W-boxes are bound by WRKY transcription factors in regulation of plant developmental processes and responses to biotic and abiotic stresses (Rushton et al. 2010; Li et al. 2015; Duan et al. 2015). Without sucrose treatment, only the shortest *IbAGP1*-VI promoter displayed absence of GUS activity and this truncated promoter does not contain a W-box (Figs. 1, 4). It suggested W-box might be required for constitutive expression of *ibAGP1*. With sucrose induction, all six truncation promoters showed increasing GUS activity in leaves, stems, and roots (Fig. 4), which may suggest that W-box, like SURE-Like box, plays an important role in sucrose-induced GUS activity in *IbAGP1* promoter. Interestingly, this shortest promoter in study was still sucrose-responsive as visible GUS staining was detected in leaves, stems, and roots in transgenic plants (Fig. 3) and the GUS activity is much higher than the WT negative control (Fig. 4 and Table S4). This result suggested the −286 to +1 region contains at least one sucrose-responsive element, which is consistent with our analysis

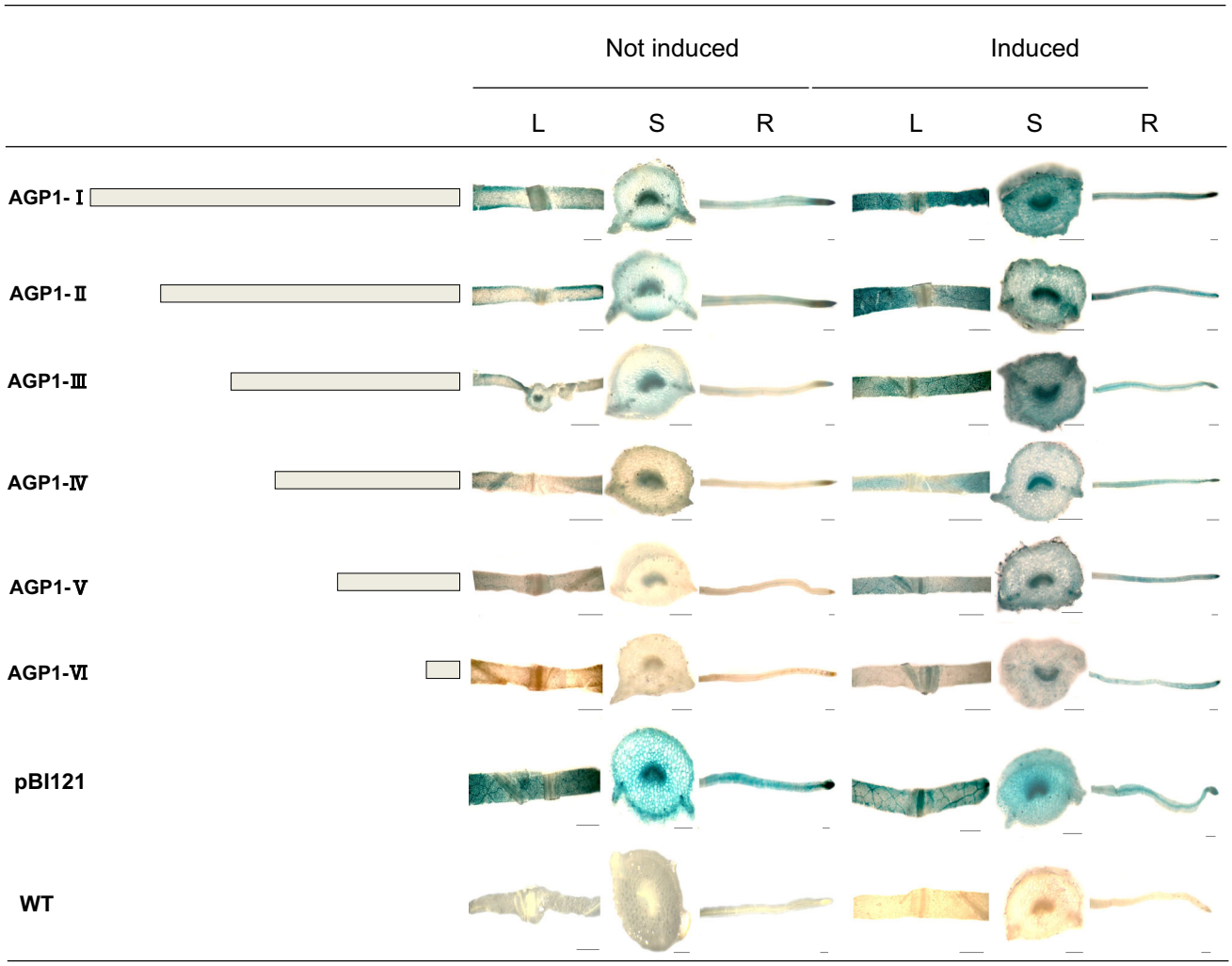

Fig. 3 Histochemical *GUS* staining of different vegetative tissues from representative transgenic tobacco plants containing different pIbAGP1::GUS constructs and vector control pBI121. *L* leaves, *S* stems, *R* roots. *WT* wild type, Induced, plantlets incubated in distilled water containing 6 % sucrose, at 25 °C for 16 h under constant light; not induced, plantlets incubated in distilled water at 25 °C for 16 h under constant light; *Bars* 1 mm

where we found there is one SURE-Like element in this region (AATAAAA, −189) (Figs. 1, 2). Another interesting observation was that sucrose-induced GUS activity in root did not change strongly as in leaves and stems, especially among *IbAGP1*-III, *IbAGP1*-IV, *IbAGP1*-V, and *IbAGP1*-VI. There are three copies of root-motif (ATATT) in −935, −400, and −245 (Fig. 1 and Table S3), which were also found in the *Agrobacterium rhizogenes rolD* promoter and 35S promoter responsible for the root specific expression (Benfey et al. 1989). When compared to other longer promoters, the sucrose-inducible expression was almost diminished in *IbAGP1*-VI in both leaves and stems, but not in roots (Figs. 1, 4). It suggests that −189 SURE-Like element and −245 root-motif that are retained in *IbAGP1*-VI may collaboratively regulate the sucrose-inducible expression in roots.

Transient expression analysis of the candidate *cis* elements with a synthetic promoter::GUS fusion construct

In the *IbAGP1* promoter, we found five SURE-Like elements and four W-boxes. To verify whether these elements function in vivo, we conducted transient expression assays in tobacco plants with a *GUS* reporter gene driven by tandem repeats of the two *cis* elements, respectively. The synthetic promoter also contains a minimal 35S promoter in between the *cis* element repeats and the *GUS* gene. The construct with the GUS gene driven by the minimal 35S promoter only was used as a negative control, and the construct with an intact 35S promoter was used as a positive control (Fig. 5). Tobacco leaves with petioles that transiently expressed the reporter constructs were detached

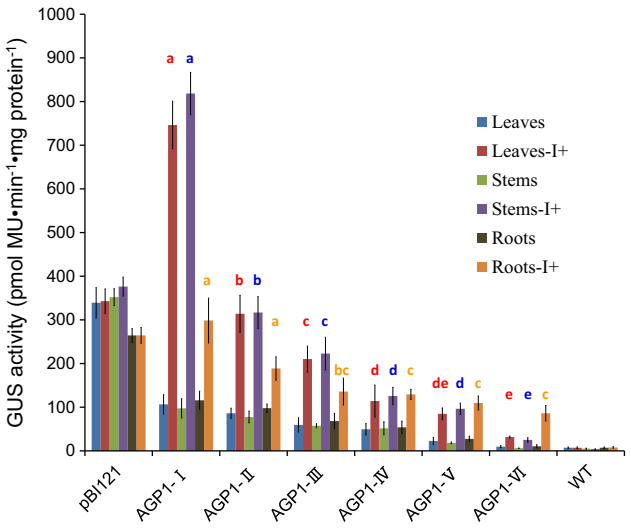

Fig. 4 Quantitative measurement of the *GUS* enzyme activity in different vegetative organs of transgenic plants containing different *pIbAGP1*::*GUS* constructs. *GUS* activity was determined using protein extracts from leaves (L), stems (S) and roots (R) of transgenic plants carrying upstream deletions of the *IbAGP1* promoter fused to *GUS* gene. GUS activity was also measured in extracts from non-transformed WT plants as a control. "I+" indicates samples were induced by sucrose. The GUS activity calculated of Supplemental Table S4. *Error bars* represent standard deviation (SD) of three replicates. Statistical significance between induced transgenic samples with different constructs in the same tissue is indicated by *different letters*. The *letters* are marked in *different colors* to indicate different tissues. (Duncan's multiple range tests, $P < 0.05$) (color figure online)

from the plant at the end of the dark period and incubated for 16 h in a solution containing 6 % sucrose. Potential activation of these synthetic promoters, along with the minimal 35S promoter and intact 35S promoter controls, was visualized by GUS staining (Fig. 5).

In the presence or absence of exogenous sucrose, we consistently observed homogenous and strong GUS expression when it was driven by an intact 35S promoter, but we did not observe GUS signals on the leaves transformed with the minimal 35S promoter::GUS construct, which was comparable to the WT control (Fig. 5). Interestingly, we found different expression profiles regarding the two *cis* elements assayed here. Both SURE-Like and W-box *cis* elements appeared to drive constitutive GUS expression, albeit weakly. Importantly, both elements also showed strong GUS induction upon the treatment of exogenous sucrose (Fig. 5). In particular, leaf samples for the 4X SURE-Like element 35Sm promoter consistently show GUS staining level that was higher or at least on par with the strong 35S promoter. The results revealed that the SURE-Like element is involved in sucrose-mediated activation of gene expression, which is consistent with the previous study in Arabidopsis and carrot taproots (Kwak et al. 2006). The involvement of the W-box in sucrose inducibility that we found here is similar to the previous finding in Arabidopsis and sweet potato (Nagata et al. 2012).

Discussion

AGPase catalyzes plant starch synthesis in the first critical step, and the sucrose content has been strongly correlated with AGPase activation. It is generally accepted that gene expression in source tissues (photosynthetic tissues) can be triggered by sugar starvation and, conversely, inactivated by high sugar content, which induces the activation of gene transcription in sink tissues with the synthesis of starch as well as other carbohydrates and storage proteins. To date, the sucrose inducibility characteristics of the AGPase large or small subunit genes were determined by assessing the transcript levels by means of Northern blot analyses (La Cognata et al. 1995; Li et al. 2002; Muller-Rober, et al. 1990; Sokolov et al. 1998). Previous studies have shown that small subunit of AGPase, the *IbAGP1* isoform, was expressed in the storage root, leaf, and stem tissues of sweet potato, and the *IbAGP1* transcript was strongly induced by applying sucrose exogenously to detached leaves (Bae and Liu 1997). The *IbAGP1* promoter is active during the later stages of storage root development. Transient expression analyses on transverse storage root sections revealed that the *IbAGP1* promoter directed strong expression in the sweet potato storage roots, which was positively correlated with endogenous sucrose contents (Kwak et al. 2006). Further analyses of the *IbAGP1* promoter and its transit peptide suggested that the *IbAGP1* promoter directed GUS expression in almost all tissues including rosette leaf, inflorescence stem, inflorescence, cauline leaf and root. These results revealed that *IbAGP1* promoter activity is constitutive (Kwak et al. 2007).

In this study, we report a more detailed expression profile of the *IbAGP1* gene promoter in transgenic tobacco through promoter truncation analysis with the *GUS* gene as a reporter. We investigated the function and sucrose-dependent induction mediated by putative *cis*-acting elements that were identified in the *IbAGP1* promoter region. The *IbAGP1* promoter contains diverse *cis*-acting elements involved in leaf-, shoot-, seed-, root-, nodule-, flower-, meristem- , and vascular tissue-specific expression. Combined with its 311 bp transit peptide (TP), the *IbAGP1* promoter is a strong constitutive foreign gene expression system for transgenesis in dicot plants (Kwak et al. 2007). Without the TP sequence, the full-length promoter of the *IbAGP1* gene (−1913 to +75) investigated in present study showed a relatively low level of constitutive expression, when compared to the 35S promoter (roughly one-third of the 35S promoter's activity in leaves and stems and half in root). By applying exogenous sucrose, there was seven- to

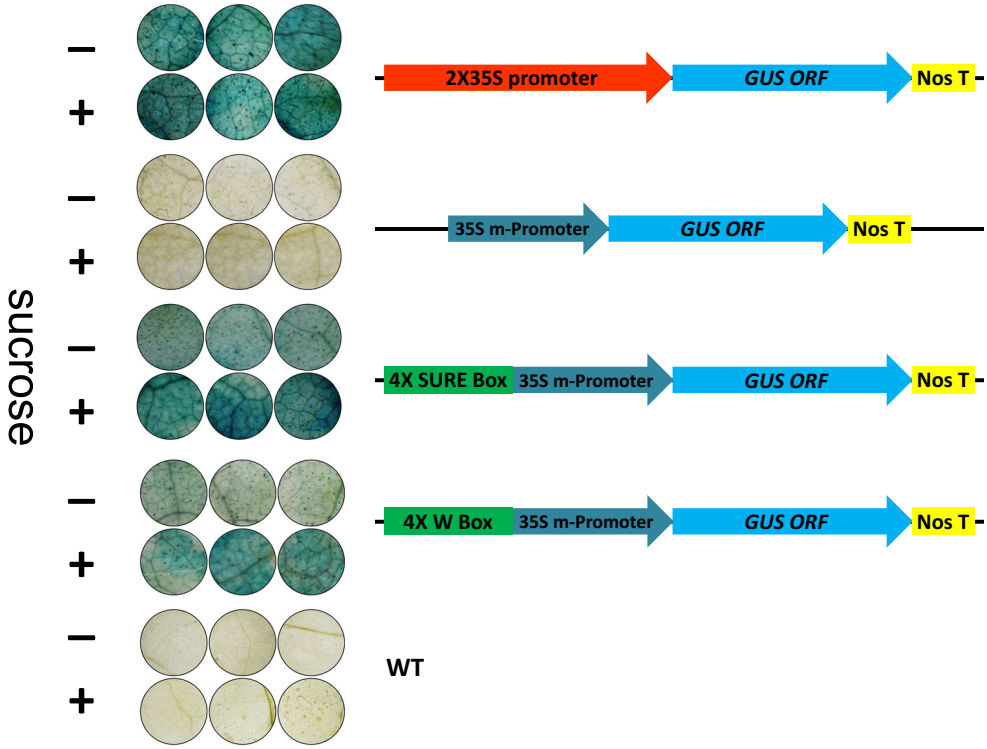

Fig. 5 Comparison the effect of putative cis-acting elements in ibAGP1 promoter on GUS expression activity in tobacco transient assays. *SURE-Like* potential sucrose responsive element, *W-box* WRKY protein-binding element, *35S m-Promoter* −46 bp 35S minimal promoter, *Sucrose* − no sucrose induce, *Sucrose* + 6 % sucrose solution induce. The experiment was repeated three times with similar results

eightfold increase of expression in leaves and stems, and threefold increase in root, which is almost two times of that of the 35S promoter. With the 5′- truncation of the *IbAGP1* promoter, the expression efficiency of the *GUS* gene was reduced accordingly. However, the sucrose-inducible expression of all other *IbAGP1*-X promoters was still significantly elevated by two or three times in all the tissues. No difference was observed for the constitutive expression levels among all three examined tissues for each promoter construct (Fig. 4).

In plant sugar signaling, several different types of *cis* elements had been identified from sugar-regulated plant promoter such as G-box (Giuliano et al. 1988), SP8 motif (Ishiguro and Nakamura 1992), SURE (Grierson et al. 1994), B-box (Grierson et al. 1994), and TGGACGG element (Maeo et al. 2001). The SP8 motif (ACTGTGTA, SP8a; TACTATT, SP8b) is present in the promoters of sporamin (SPO-A1 and SPO-B1) and beta-amylase genes from sweet potato tuberous roots (Ishiguro and Nakamura 1992). The SP8 motif was bound by the transcription factor SPF1, which belongs to WRKY family and functions as a repressor (Ishiguro and Nakamura 1994). The 9-bp SURE, AATACTAAT and AATAGAAAA, were first identified from patatin promoter in potato (Grierson et al. 1994). It was shown that a sucrose-inducible WRKY protein, SUSIBA2, could bind to the SURE and W-box elements as an activator (Sun et al. 2003). However, there was no identical SURE or SP8 sequences in the *IbAGP1* promoter. In a previous study, sequence analysis of the *IbAGP1* promoter identified four putative SURE-Like elements (Kwak et al. 2006). But it remains unclear whether these SURE-like fragments in the *IbAGP1* promoters truly function as SUREs. In this study, we identified five potential SURE-Like elements (AATAAATAAA, −1273 and −1239; AATAAAAAA, −681; AATAAAA, −610, and −189) in *IbAGP1* promoter (Fig. 1 and Table S3). All the six *IbAGP1*-X promoter-driven GUS expression constructs showed sucrose-dependent activation, which suggested that the five SURE-Like elements likely played a role in sucrose responsiveness. To further dissect the involvement of these SURE-Like elements in sucrose-inducibility, AATAAATAAA element was tandem repeated for four times and combined with a 46-bp 35-s minimal promoter to drive the GUS expression. Without the influence of other positive or negative *cis*-acting elements, SURE-Like element indeed strongly up-regulated expression of the *GUS* gene by an exogenous sucrose treatment, functioning just like the SUREs from the potato patatin promoter. It clearly demonstrated that sucrose-induced expression of the *IbAGP1* promoter in tobacco could be attributed to the SURE-Like elements identified within this promoter (Fig. 5). Unlike the WT tobacco leaves, the pSURE-Like tobacco leaves showed visible GUS staining without exogenous sucrose treatment. The results suggest that SURE-Like elements may play a role in constitutive gene expression of *IbAGP1*. However, we cannot rule out the

possibility that the SURE-like elements may be solely involved in sucrose response and the GUS expression that we observed in the absence of the exogenous sucrose could be induced by endogenous sucrose in the leaf tissues.

Interestingly, by truncating ~300 bp from 5′ end of the full-length *IbAGP1* promoter, the sucrose-induced expression lost a half (Fig. 4), which pointed to the important role of one W-box and other *cis*-acting elements like CAAT enhancer in the region (from −1913 bp to −1598 bp). It has been found that a sucrose-inducible WRKY protein, SUSIBA2, binds to the SURE and W-box element (Sun et al. 2003). A WRKY transcription factor from Arabidopsis, AtRKY20, was proven to enhance the expression of Arabidopsis AGPase large subunit gene, *ApL3*, by directly binding to the *ApL3* promoter and activating the transcription. Transient expression experiments demonstrated that AtWRKY20 also activated the promoter of sweet potato AGPase small subunit gene, *IbAGP1,* by directly interacting with the region between positions −623 and −490 in the *IbAGP1* promoter (Nagata et al. 2012). Moreover, the W-box of the *IbAGP1* promoter bound by AtWRKY20 activator might be an enhancer in sweet potato (Nagata et al. 2012). There are four W-box elements found in the *IbAGP1* promoter, which are located in −1985 (*IbAGP1*-I), −1434 (*IbAGP1*-II), −750 (*IbAGP1*-III, *IbAGP1*-IV), and −578 (*IbAGP1*-V). Only the shortest *IbAGP1*-VI promoter does not contain a W-box, which might explain why the *IbAGP1*-VI transgenic tobacco plants have no GUS activity without sucrose treatment. In this study, we found the W-box tandem promoter also showed sucrose-inducible activation in transient tobacco leaves, which further demonstrated the W-box is important for sucrose-inducible activation of the *IbAGP1* promoter.

The *IbAGP1* promoter contained 121 leaf-, 89 shoot-, 70 seed-, 27 root-, 16 nodulin-, 1 flower-, 1 meristem- , and 1 vascular-tissue related elements. In addition, leaf cell-specific motifs including 33 mesophyll cell-, 23 chlorophyll-, 7 guard cell-, and 3 plastid-specific elements were found (Kwak et al. 2007). Five endosperm-, 3 embryo-, 6 petal epidermis-, and 26 pollen-specific elements were also identified (Kwak et al. 2007). These results suggest that the *IbAGP1* promoter activity might be constitutive (Kwak et al. 2007). However, in present study, sucrose inducibility of the *IbAGP1* promoter varied between aboveground tissues (leaves and stems) and ground tissues (roots). These data also indirectly suggest some of the promoter elements indeed mediate differential expression among aboveground and underground tissues. For example, when comparing the sucrose-inducible GUS expression of *IbAGP1*-III, *IbAGP1*-IV, *IbAGP1*-V, and *IbAGP1*-VI, we did not find significant differential gene expression in roots (Fig. 4). The SURE-box element closest to TSS (AATAAAA, −189) seems to be the only element responsible for sucrose inducibility in roots within the length of −1298 bp to +1 (Fig. 4). This indicates the additional four SURE-Like boxes in this region may only be involved in sucrose inducibility in aboveground tissues if at all. Our transient GUS reporter assays have demonstrated that one SURE-like element (AATAAATAAA, −1273 and −1239) is indeed involved in sucrose inducibility in one aboveground tissue, leaves (Fig. 5).

In conclusion, our study has showed that the *IbAGP1* gene promoter from sweet potato directs the *GUS* reporter gene in the storage root, leaf, and stem tissues in tobacco. Although the expression level is only one-third in aboveground tissues and a half in underground tissues of that of the 35S promoter, the expression can be boosted to a much higher level by applying exogenous sucrose. By utilizing the *IbAGP1* promoter, we have established a tobacco expression system with a "constitutive and tunable" property. We have mapped some putative promoter elements, such as SURE-Like and W-box, for their roles in differential inducibility among tissues by sucrose. Using a transient assay, we confirmed both SURE-like and W-box are indeed mediating sucrose-induced gene expression in tobacco leaves. Further analyses of these elements will provide an insight into the signaling pathway involved in the regulation of starch biosynthesis in storage tissues. And we could potentially improve this expression system in the future for its application in biotechnology.

Author contribution statement X.L. ZH., Q.L., Y.P.Q., and Y.ZH. conceived and designed the experiments. X. L.ZH., Q. L., D.Q. L, L.L. Z., K.Y. ZH., S.X. Y., Z.Y. X., and X. T. performed the experiments. X.L. ZH., Q. L., K.J. D., Y.P. Q., and Y. ZH. analyzed the data. X.L. Z., Q. L., D.Q. L, L.L. Z., S.X. Y., Z.Y. X., and X. T. contributed reagents/materials/analysis tools. X.L. ZH., Q. L., Y.P. Q., and Y. ZH. wrote the paper.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict interests.

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