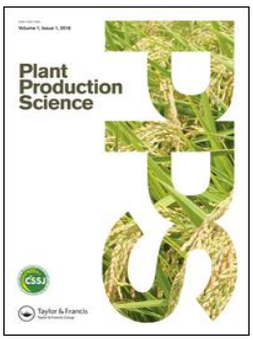

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Activation of ADP-Glucose Pyrophosphorylase Gene Promoters by a WRKY Transcription Factor, AtWRKY20, in *Arabidopsis thaliana* L. and Sweet Potato (*Ipomoea batatas* Lam.)

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Abstract: ADP–glucose pyrophosphorylase (AGPase) catalyzes the first limiting step in starch biosynthesis in plants. However, the direct transcriptional activator of the AGPase genes has not yet been determined. We have isolated a WRKY transcription factor cDNA, *AtWRKY20*, from *Arabidopsis thaliana* and purified the corresponding protein. Transient expression of *AtWRKY20* by particle bombardment enhanced expression of the promoter of *ApL3*, encoding a sugar-inducible AGPase large subunit gene of *A. thaliana*, in leaves of *A. thaliana*. AtWRKY20 bound to the *ApL3* promoter *in vitro*. The expression of *AtWRKY20* was strongly induced by sucrose or, to a lesser extent, by mannitol, and the expression pattern of the *ApL3* gene mimicked that of the *AtWRKY20* gene. Transient expression experiments demonstrated that AtWRKY20 also activated the promoter of Koganesengan *ibAGP1* encoding an AGPase small subunit gene of sweet potato var. Koganesengan. A 5'−end deletion analysis revealed a negative regulatory region from −1371 to −641 and a positive regulatory region from −640 to −180 in the Koganesengan *ibAGP1* promoter. AtWRKY20 interacted directly with the region between positions −623 and −490 in the Koganesengan *ibAGP1* promoter. These results suggest that AtWRKY20 functions directly as a transcriptional activator of the *ApL3* promoter and regulates the expression of *ApL3* induced by sucrose or osmoticum in *A. thaliana*. Moreover, AtWRKY20 can enhance the expression of the Koganesengan *ibAGP1* promoter directly in sweet potato.

Key words: ADP-glucose pyrophosphorylase, *Arabidopsis thaliana* L., Promoter, Sweet potato cv. Koganesengan, Transactivation, WRKY.

For further improvement of crop productivity, a higher starch content in the sink organs is an important breeding target. Direct introduction of genes by genetic engineering seems an attractive and quick method for increment in the starch content. ADP-glucose pyrophosphorylase (AGPase, EC 2.7.7.27), a key enzyme in starch biosynthesis, catalyses the conversion of glucose-1-phosphate to ADP-glucose, which serves as a direct substrate for starch synthesis (Preiss, 1984). Nakatani and Komeichi (1992) reported a positive correlation between AGPase activity and the starch content in the tuberous roots of sweet potato. Three mechanisms are known to regulate AGPase activity: (i) transcriptional regulation (Müller-Röber et al., 1990; Sokolov et al., 1998; Akihiro et al., 2005; Nagata and Saitou, 2009); (ii) allosteric regulation, via glycerate-3-phosphate and inorganic phosphate (Sowokinos and Preiss, 1982); and (iii) post-translational redox modification in response to sugars (Tiessen et al., 2002; Hendriks et al., 2003; Michalska et al., 2009). AGPase is a heterotetramer in higher plants and is composed of two large and two small subunits (Morell et al., 1987). Each subunit is encoded by a different gene. Six genes encode proteins with homology to AGPase in the *Arabidopsis thaliana* genome (Crevillén et al., 2005). Two of these genes encode small subunits, *ApS1* (locus number At5g48300) and *ApS2* (locus number At1g05610), and the other four encode

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Abbreviations: AGPase, ADP-glucose pyrophosphorylase; ASML2, activator of *Spo^{min}-LUC2*; CaMV, cauliflower mosaic virus; GST, glutathione S–transferase; His, histidine; LUC, luciferase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SURE, sucrose–responsive element; UTR, untranslated region.

large subunits, *ApL1* to *ApL4* (locus numbers At5g19220, At1g27680, At4g39210, and At2g21590, respectively).

In sweet potato (*Ipomoea batatas* Lam.), five cDNA clones encoding two small subunits and three large subunits of AGPase have been cloned and designated *ibAGP1* and *ibAGP2* (Bae and Liu, 1997; Noh et al., 2004), and *ibAGPL1-1*, *ibAGPL1-2*, and *ibAGPL1-3* (Harn et al., 2000), respectively. Both *ibAGP1* and *ibAGP2* are expressed in the tuberous root, leaf, and stem tissues of sweet potato. The levels of *ibAGP1* mRNA can be enormously increased by applying sucrose exogenously to detached leaves, whereas the transcript level of *ibAGP2* remains almost unaffected by this treatment (Bae and Liu, 1997). Moreover, the *ibAGP1* promoter is upregulated by increasing the endogenous sucrose contents of tuberous roots, whereas the *ibAGP2* promoter is downregulated (Kwak et al., 2006).

Because a single transcription factor is frequently involved in expression of multiple genes for a metabolic pathway, it might be possible to modulate biological processes including numerous enzymatic reactions, such as starch biosynthesis, by using a single transcription factor. *WRKY* genes belong to a gene superfamily of transcription factors that are involved in the regulation of various biological processes, including pathogen defense, senescence, and development (Ülker and Somssich, 2004). *WRKY* gene products have either one or two WRKY domains, each containing a 60-amino acid region with a core sequence, WRKYGQK, at its N-terminal end and a novel zinc finger-like motif. WRKY proteins bind to the DNA sequence motif 5'-(T)TGAC(C/T)-3', known as the W-box (Eulgem et al., 2000). WRKY proteins can activate or repress transcription (Rushton et al., 2010). A WRKY transcription factor in barley, SUSIBA2, has been shown to bind to W-box and sucrose-responsive elements (SUREs) (Grierson et al., 1994) in the promoter of the *isoamylase1* gene as an activator (Sun et al., 2003). A WRKY transcription factor in sweet potato, SPF1, is able to bind to the sugar-responsive sporamin and β-amylase promoters (Ishiguro and Nakamura, 1994), and this suggests that SPF1 acts as a repressor (Rook et al., 2006). In *A. thaliana*, Masaki et al. (2005) reported an activator of *Spo^{min}-LUC2* (ASML2; locus number At3g12890), which is a protein belonging to CCT (Constans, Constans-like, TOC1) domain proteins, functions as a transcriptional activator of *ApL3*, and expression of *ASML2* gene is enhanced by sugars in *A. thaliana*. However, DNA-binding transcriptional activators that regulate the expression of the AGPase genes in response to sugar have not been identified.

Analysis of *A. thaliana* genome sequences revealed 74 *WRKY* genes (Ülker and Somssich, 2004), although the nuclear genome size of *A. thaliana* is the smallest among known flowering plants (Leutwiler et al., 1984). Sweet potato is a hexaploid having much larger genome size than *A. thaliana*, but WRKY transcription factors except SPF1 have not been reported in sweet potato. Our ultimate objective is to identify a novel transcriptional activator of AGPase gene promoters in response to sugar in sweet potato. As an approach to this goal, we first used *A. thaliana* to identify WRKY transcription factors that activate the expression of the AGPase genes in plants, and then we tried to apply the *A. thaliana* findings to sweet potato. Here, we studied a WRKY transcription factor, AtWRKY20 (locus number At4g26640), functions directly as a transcriptional activator of the *ApL3* promoter in response to sugars in *A. thaliana*, and examined functional and physical interactions with AtWRKY20 and the promoter of *ibAGP1* to assess a possible function of AtWRKY20 as a transcriptional activator in sweet potato.

Materials and Methods

1. Plant materials

Seeds of *Arabidopsis thaliana* L. ecotype Columbia were sown on rockwool and grown by hydroponics in a growth chamber under a 12 hr photoperiod with a light intensity of 100 μmol m^{-2} s^{-1}, a relative humidity of 60%, and at 20°C. The hydroponic medium contained 2.5 mM potassium phosphate buffer (pH 5.5), 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 5 mM KNO_3, 50 μM iron(III) monosodium ethylenediaminetetraacetate, 10 μM NaCl, 70 μM H_3BO_4, 14 μM $MnCl_2$, 1 μM $ZnCl_2$, 0.5 μM $CuSO_4$, 0.01 μM $CoCl_2$, and 0.2 μM Na_2MoO_4.

Sweet potato plants (*Ipomoea batatas* Lam. cv. Koganesengan) were grown in a growth chamber under a 12 hr photoperiod with a light intensity of 250 μmol m^{-2} s^{-1}, a relative humidity of 70%, and at 25°C as described previously (Saitou et al., 1997).

2. Construction of vectors for the transient expression assay

To make a reporter construct in *A. thaliana*, we excised the ubiquitin promoter and LR clonase recombination cassette of the pANDA-mini vector (Miki and Shimamoto, 2004), and inserted a Gateway RfA cassette (Invitrogen, Carlsbad, CA, USA) and a *Renilla LUCIFERASE* gene (*hRluc*) of the phRG-B vector (Promega, Madison, WI, USA) into the *Hin*dIII/*Sac*I site of the pANDA-mini vector, generating *RfA-hRluc*. A total of 873 bp of the *ApL3* 5' flanking region [761 bp of promoter region plus 112 bp of 5' untranslated region (UTR)] was amplified from genomic DNA of *A. thaliana* by polymerase chain reaction (PCR) using two primers as follows: the forward primer 5'-CACCC GCCTACAGCCCATTTGACAAGTTT-3', and the reverse primer 5'-CGTTTGAAATCGAGGGAAGACCAAGA-3'. The fragment of the *ApL3* promoter was ligated into the pENTR^{TM}/D-TOPO$^{®}$ vector (Invitrogen, Carlsbad, CA, USA), and was then inserted into *RfA-hRluc* with an LR reaction (Invitrogen).

Genomic DNA was extracted from tuberous roots of

sweet potato cv. Koganesengan as described by Murakami et al. (1986), with minor modifications. A total of 1415 bp of the Koganesengan *ibAGP1* 5' flanking region (*p-1371*; 1371 bp of promoter region plus 44 bp of 5' untranslated region) was amplified from genomic DNA of tuberous roots by PCR using two primers as follows: the forward primer 5'-CCCAAGCTTGGGCAACAGCCAGCATTAGGA TGTG-3' which incorporated a *Hin*dIII site (underlined) to the 5'-end of the PCR product, and the reverse primer (RP) 5'-CGGGATCCCGCTCTCTGCGGACTTTGGAG-3' which added a *Bam*HI site (underlined) in its 3'-end. PCR amplification was carried out for 35 cycles under the following standard conditions: denaturation (98ºC for 10 s), annealing (65ºC for 30 s), extension (68ºC for 2 min) using KOD-Plus-Ver.2 (TOYOBO, Osaka, Japan). DNA sequencing was performed at Macrogen Inc. (Seoul, Korea). Only three nucleotides in the Koganesengan *ibAGP1* promoter (accession number AB535526) were substituted as compared with the *ibAGP1* promoter of cv. Yulmi (Kwak et al., 2006), at nucleotide positions −922, −453 and −266 of the Koganesengan *ibAGP1* promoter. To make constructs for the transient expression vectors in sweet potato, the *hRluc* gene of the phRG-B vector (Promega, Madison, WI, USA) was ligated to the *Bam*HI/*Sac*I site of pBI221 (Clontech, Palto Alto, CA, USA), replacing the *β-GLUCURONIDASE* gene, and generating *pCaMV35S-hRluc*. Deletion fragments of the Koganesengan *ibAGP1* promoter, *p-640* and *p-179* (640 and 179 bp, respectively, of the promoter region plus 44 bp of the 5' UTR) were amplified using as forward primers that incorporated a *Hin*dIII site (underlined) to their 5'-ends: 5'-CCCAAGCTTGGGGCTCATAACTTACTAGTTCAGA-3' and 5'-CCCAAGCTTGGGGTATACCCCACCCTGATTTT TG-3', respectively. The common reverse primer used was RP as mentioned in *p-1371*. *p-1371*, *p-640*, and *p-179* were inserted each into the *Hin*dIII/*Bam*HI site of *pCaMV35S-hRluc*, replacing the cauliflower mosaic virus (CaMV) 35S promoter. The resultant plasmids were named *p-1371-hRluc*, *p-640-hRluc*, and *p-179-hRluc*, respectively.

To make effector constructs, the ubiquitin promoter and LR clonase recombination cassette of the pANDA-mini vector (Miki and Shimamoto, 2004) were excised, and a promoter cassette (E12Ω) of pBE2113-GUS (Mitsuhara et al., 1996) and a Gateway RfA cassette (Invitrogen, Carlsbad, CA, USA) were inserted into the *Hin*dIII/*Sac*I site of the pANDA-mini vector, generating *E12Ω-RfA*. Full-length cDNAs of *AtWRKY20* (locus number At4g26640) and *ASML2* (locus number At3g12890) were amplified from RIKEN Arabidopsis full-length cDNA clones (resource numbers pda01430 and pda08813, respectively; Seki et al., 1998; Seki et al., 2002). The cDNAs were ligated into pENTR™/D-TOPO® vectors (Invitrogen), and were then inserted into *E12Ω-RfA* with an LR reaction (Invitrogen).

To construct an internal control vector, a fragment containing the 5'-upstream sequence of the CaMV 35S promoter (−90 to −1; *−90p35S*) and the 5'-untranslated sequence of tobacco mosaic virus (*Ω*) of pBE2113-GUS were fused to the firefly *LUCIFERASE* gene (*luc*$^+$) of the pGL3 basic vector (Promega). The *−90p35S-Ω-luc*$^+$ fragment was then inserted into the *Hin*dIII/*Sac*I site of the pANDA-mini vector, replacing the ubiquitin promoter and the LR clonase recombination cassette. The resulting plasmid was named *−90p35SΩ-luc*$^+$. The *−90p35SΩ* promoter lacks potential sites for binding to WRKY.

3. Transient expression assay

DNA-coated microparticles were prepared by the $CaCl_2$/spermidine method as described by Sanford et al. (1993). A 500 ng aliquot of the reporter construct and 400 ng of *−90p35SΩ-luc+*, and if needed, 250 ng of the effector construct, were mixed with 375 μg of gold particles (1.0 Micron Gold, Bio-Rad Laboratories, Hercules, CA, USA) in the presence of 0.1 M spermidine and 2.5 M $CaCl_2$. Uppermost mature leaves of 11-day-old *A. thaliana* plants were bombarded from the reverse side with plasmid-coated gold particles (375 μg per bombardment) using a biolistic gun device (PDS-1000/He; Bio-Rad Laboratories), with the stopping screen positioned 3 cm below the rupture discs, the target leaves positioned 3 cm below the stopping screen, and a helium pressure of 4.5 MPa. After 2 hr of incubation, bombarded leaves were cut from plants, and submerged in 100 mM sucrose solution for 19 hr at 20ºC in the dark.

In sweet potato, a 400 ng aliquot of the *p-1371-hRluc* plasmid and 400 ng of *−90p35SΩ-luc+*, and if needed, 20 ng of the effector construct, were mixed with 375 μg of gold particles (1.0 Micron Gold, Bio-Rad Laboratories) in the presence of 0.1 M spermidine and 2.5 M $CaCl_2$. Petioles with the intact leaf attached, were cut from plants grown in the growth chamber and the cut edges of these leaf-petiole cuttings were submerged in distilled water at 25ºC in the dark for 24 hr, and then in 150 mM sucrose solution for another 24 hr. Next, petioles were transversely cut into 2 mm-thick sections and were positioned on Murashige and Skoog media (Murashige and Skoog, 1962) in petri dishes. A biolistic gun device (PDS-1000/He; Bio-Rad Laboratories) was used to deliver plasmid-coated gold particles (375 μg per bombardment), with the stopping screen positioned 3 cm below the rupture disc, the target tissue positioned 3 cm below the stopping screen, and a helium pressure of 9.3 MPa. The bombarded cross-sections were then incubated for 18 hr at 25ºC in the dark.

Bombarded leaves or petioles were homogenized with a pestle in an ice-cold mortar, and were measured for *Renilla* and firefly luciferase (LUC) activities with the Dual-Luciferase® reporter assay system (Promega) according to

the manufacturer's instructions, using a luminometer (GloMax™ 20/20n; Promega) to assess chemiluminescence. The *Renilla* LUC activity values were normalized to firefly LUC activity values for differences in transformation efficiency.

4. Production of the recombinant AtWRKY20

pET-21a(+) (Novagen, Madison, WI, USA) and pGEX KG (GE Healthcare UK Ltd., Little Chalfont, UK) were digested with *Hin*dIII and *Pst*I, and a fragment of pET-21a(+) containing the histidine (His) tag coding sequence was ligated into a pGEX KG fragment containing the *GLUTATHIONE S-TRANSFERASE* (*GST*) gene. The resultant plasmid was named pGEX KG ori-Hisx6.

The sequence encoding *AtWRKY20* was amplified by PCR using oligonucleotide primers (5'-CACCGTGGGGAT TCCTTCCTTTTACTTATATGAC-3' and 5'-CGTCCCA TTCCTAACCTAAGAAGATC-3') and was cloned into pGEX KG ori-Hisx6 to produce a protein composed of an N-terminal GST tag, a thrombin cleavage site, and a C-terminal His tag fused to AtWRKY20. The resulting plasmid was introduced into *Escherichia coli* BL21 (GE Healthcare UK Ltd.). The recombinant protein was induced with 0.1 mM isopropyl-β-D-thiogalactoside at 37ºC. The recombinant protein was first purified with Glutathione Sepharose 4B according to the protocol provided by GE Healthcare UL Ltd., then, its GST was excised with restriction-grade Thrombin (Novagen), and the His-tagged AtWRKY20 protein was purified on TALON® metal affinity resin according to the manufacturer's instructions (Clontech).

5. Electrophoretic mobility shift assay

The electrophoretic mobility shift assay was performed with a Second-Generation DIG Gel Shift kit (Roche, Mannheim, Germany). The DNA probes used in this assay were fragments of the Koganesengan *ibAGP1* promoter spanning positions −623 to −426, −581 to −426, −567 to −426, −640 to −490, and −373 to −157, which contained SURE-like fragments (Kwak et al., 2006) and W-box core sequences (Ciolkowski et al., 2008). The purified AtWRKY20 protein (0.6 pmol) was incubated with 15 fmol of DNA probe at room temperature for 30 min, and then the samples were electrophoresed on a 6% non-denaturing polyacrylamide gel in 0.5×TBE (44.5 mM Tris base, 44.5 mM boric acid, and 1 mM ethylenediaminetetraacetic acid disodium salt) buffer. The gel was blotted on a Hybond N^+-nylon membrane (GE Healthcare UK Ltd.). The chemiluminescence emitted by Anti-Digoxigenin-AP and disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricycle [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate was imaged with a VersaDoc 5000 imaging system and Quantity One software (Bio-Rad Laboratories).

6. Reverse transcription-polymerase chain reaction (RT-PCR) experiment

Uppermost mature leaves were cut from 11-day-old plants of *A. thaliana*, and submerged in distilled water, 100 mM sucrose, or 100 mM mannnitol solution for 19 hr at 20ºC in the dark. Total RNA was isolated from the treated leaves by a method described by Nagata and Saitou (2009) with minor modification. First-strand cDNA was synthesized from 2 μg total RNA, pretreated with a Message Clean™ kit (Gen Hunter, Nashville, TN, USA), using a Takara RNA PCR kit (AMV) Ver. 3.0 (Takara Bio Inc., Otsu, Japan) with random hexamer, according to the manufacturer's instructions. Semi-quantitative RT-PCR was performed with 1/5 for *AtWRKY20* or 1/40 for *ApL3* of the first-strand reaction mix, with the following gene-specific primers: 5'-CACCGTGGGGATTCCTTT TACTTATATGAC-3' as a forward primer and 5'-CGTCCCATTCCTAACCTAAGAAGATC-3' as a reverse primer for *AtWRKY20*; 5'-TGGGAAGAGCATC GGAACTATTAAGAGT-3' as a forward primer and 5'-TTG AGGTGTTTGGCCGGAAGTTAAAAAG-3' as a reverse primer for *ApL3*. As a loading control, the 18S rRNA gene was amplified using primers, 5'-GCTTGTCTCAAAGATTA AGCCATGCATG-3' as a forward primer and 5'-TCAGGCTCCCTCCGGAATCGAA-3' as a reverse primer. The PCR conditions for *AtWRKY20* were 94ºC for 30 s, 65ºC for 30 s, and 72ºC for 2 min. The PCR conditions for *ApL3* were 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 1 min 30 s. The reaction was repeated for 13–32 cycles to obtain an appropriate amount of DNA. The cycle numbers were determined to avoid the saturation of DNA amplification. PCR products were separated on a 1.5% agarose gel, followed by 1 hr staining with ethidium bromide. The image of the stained gel was recorded with a gel image analyzer (Molecular Imager FX; Bio-Rad Laboratories). The image density of each stained PCR product was analyzed using Quantity One software (Bio-Rad Laboratories). To confirm gene-specific amplification, we cloned and fully sequenced the amplified products.

Results and Discussion

1. Effect of AtWRKY20 on the expression of the *ApL3* promoter

Using the PLACE Web Signal Scan program (http://www.dna.affrc.go.jp/PLACE/), we identified three W-box core sequences (5'-TGAC-3') that could interact with WRKY transcription factors in the *ApL3* promoter. To isolate a cDNA clone for WRKY transcription factor that regulates the expression of *ApL3*, we compared 74 WRKY proteins of *A. thaliana* with sweet potato SPF1, which belongs to the WRKY superfamily of plant transcription factors, by using the BLASTP 2.2.17 program (http://blast.genome.jp/), with the default parameters. AtWRKY20 (locus number At4g26640) shared the highest sequence

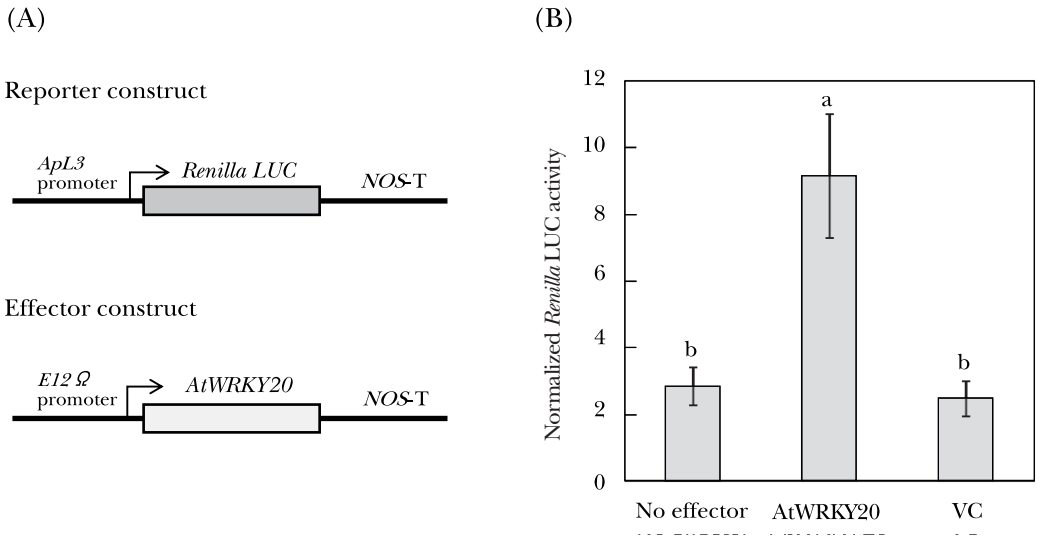

Fig. 1. Transient expression assays of the *ApL3* promoter by AtWRKY20 in *A. thaliana* leaves. (A) Schematic representation of the reporter and effector constructs. The reporter construct contains a 873-bp upstream sequence of the *ApL3* gene that controls *Renilla LUC* gene expression. In the effector constructs, expression of *AtWRKY20* gene was driven by a promoter cassette (E12Ω) of pBE2113-GUS (Mitsuhara et al., 1996). (B) Transient expression assays by co-bombardment of leaves with the reporter and effector constructs and an internal control construct containing the firefly *LUC* gene. *Renilla* LUC activity was normalized relative to the firefly LUC activity for every independent transformation. VC indicates the vector control (the empty effector construct lacking *AtWRKY20* cDNA). Values with the same letters do not differ significantly ($P<0.05$, Tukey's test). Bars indicate SD ($n=3$).

similarity with the SPF1 protein.

We examined whether AtWRKY20 modulates the transcriptional activity of *ApL3* promoter by performing transient expression experiments. Mature leaves were bombarded with either the reporter construct alone or the reporter construct in combination with the effector (Fig. 1A). Co-bombardment of the reporter construct in combination with the *AtWRKY20* construct as the effector increased *Renilla* LUC activity by nearly 3-fold compared with the activity measured with bombardment by the reporter construct alone (Fig. 1B). *Renilla* LUC activity did not vary significantly with co-introduction of the control vector. These results suggest that AtWRKY20 enhances expression of the *ApL3* promoter, although SPF1 is known as a repressor in sweet potato (Rook et al., 2006).

2. Binding of AtWRKY20 to the *ApL3* promoter

A DNA fragment that contained the *ApL3* promoter region from −134 to −27 was labeled with digoxigenin (Fig. 2A). The DNA fragment included all of three W-box core sequences (5'-TGAC-3') in the *ApL3* promoter. In an electrophoretic mobility shift assay, no binding signal was detected in reactions without protein (Fig. 2B, lane 1) or with His-tagged GST (lane 2). Addition of the AtWRKY20 protein efficiently retarded the mobility of the probe, revealing a shift band (lane 3). These results indicate that AtWRKY20 interacts directly with the *ApL3* promoter.

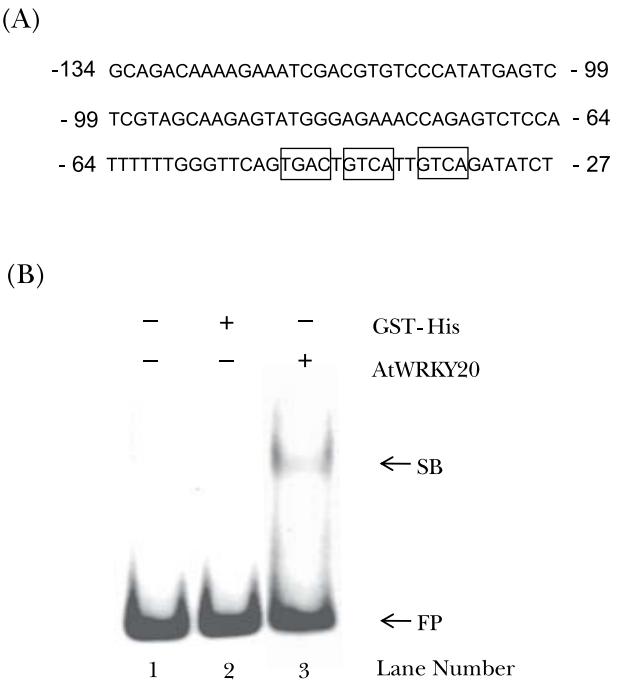

Fig. 2. Binding of AtWRKY20 to the *ApL3* promoter region. (A) DNA sequences of the *ApL3* promoter used as probes. Nucleotide numbers are on either side, with the position of the transcriptional start site designated +1 (Noh et al., 2004). Boxes indicate W-box core sequences. (B) Electrophoretic mobility shift assays of the *ApL3* promoter region incubated with recombinant His-tagged GST or AtWRKY20 protein. Arrows indicate the positions of the shift band (SB) and the free probes (FP).

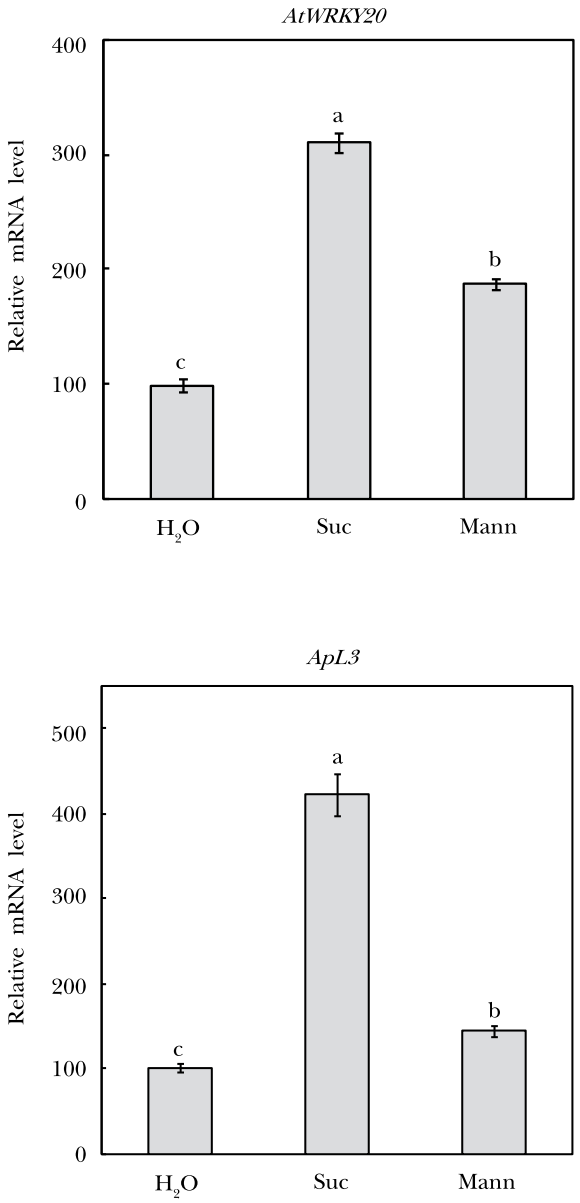

Fig. 3. Steady-state levels of mRNA for *AtWRKY20* and *ApL3* in detached leaves of *A. thaliana* after treatment with sucrose or mannitol. Total RNA was isolated from the detached leaves that had been submerged in distilled water (H_2O), 100 mM sucrose (Suc), or 100 mM mannitol (Mann) for 19 hr in the dark. The mRNA levels were analyzed by RT-PCR in the exponential range of amplification. The mRNA levels of *AtWRKY20* and *ApL3* were quantified from images of gel electrophoresis, and normalized relative to 18S rRNA levels. Values with the same letters do not differ significantly ($P < 0.05$, Tukey's test). Bars indicate SD (n=3).

3. Effects of sugars on the expression of *AtWRKY20* and *ApL3* genes

The expression of *AtWRKY20* was strongly induced by sucrose or, to a lesser extent, by mannitol (Fig. 3). The sucrose-induced increase in expression levels of *AtWRKY20* appeared independent of changes in osmotic pressure, as mannnitol could not mimic the effect of sucrose. Sucrose induced strong expression of *ApL3* (Fig. 3). Mannitol was also able to induce the *ApL3* gene expression, but slightly. The expression pattern of the *ApL3* gene mimicked that of the *AtWRKY20* gene, suggesting that AtWRKY20 is a transcriptional activator in modulating sucrose- or osmolality- induced expression of the *ApL3* gene.

4. Effects of AtWRKY20 and ASML2 on the expression of the Koganesengan *ibAGP1* promoter

Co-bombardment of the reporter construct with the *AtWRKY20* construct as the effector increased *Renilla* LUC activity by nearly 4-fold compared with the activity measured with bombardment by the reporter construct alone (Fig. 4). ASML2 failed to activate the reporter *Renilla LUC* gene, and *Renilla* LUC activity did not vary significantly with co-introduction of the control vector. These results indicate that AtWRKY20 enhances expression of the Koganesengan *ibAGP1* promoter in sweet potato, but ASML2 does not.

Transcript levels of both a single functional *A. thaliana* small subunit gene, *ApS1*, and the *A. thaliana* large subunit gene, *ApL3*, are strongly increased when sucrose is fed to detached leaves (Sokolov et al., 1998). However, *ApS1* showed a sucrose-insensitive expression pattern under normal physiological conditions when mature *A. thaliana* plants were irrigated with sucrose-containing Murashige and Skoog medium, whereas *ApL3* was induced by irrigation with the sucrose-containing medium (Crevillén et al., 2005). These observations suggest that the sucrose-mediated regulation mechanism for the expression of the small subunit gene may be different from that for the large subunit gene. The promoter of *ibAGP1* encoding the AGPase small subunit of sweet potato was activated by AtWRKY20 (Fig. 4), which is a transcriptional activator of *ApL3* promoter in the modulation by sucrose signaling in *A. thaliana*. On the other hand, ASML2, which enhances the expression of *ApL3*, did not activate the *ibAGP1* promoter. It is likely that the distinction in the sucrose-mediated regulation mechanism between small subunit and large subunit genes includes the difference in effects on the expression of small subunit gene promoter between AtWRKY20 and ASML2.

5. 5'-end deletion analysis of the Koganesengan *ibAGP1* promoter

Deletion up to position −641 from −1371 of the Koganesengan *ibAGP1* promoter enhanced *Renilla* LUC activity (Fig. 5), suggesting the presence of a negative regulatory element in the region between positions −1371 and −641. Further deletion up to position −180 in the construct dramatically reduced *Renilla* LUC activity compared with the −640 bp promoter fragment construct. This indicates that there is a positive regulatory element in

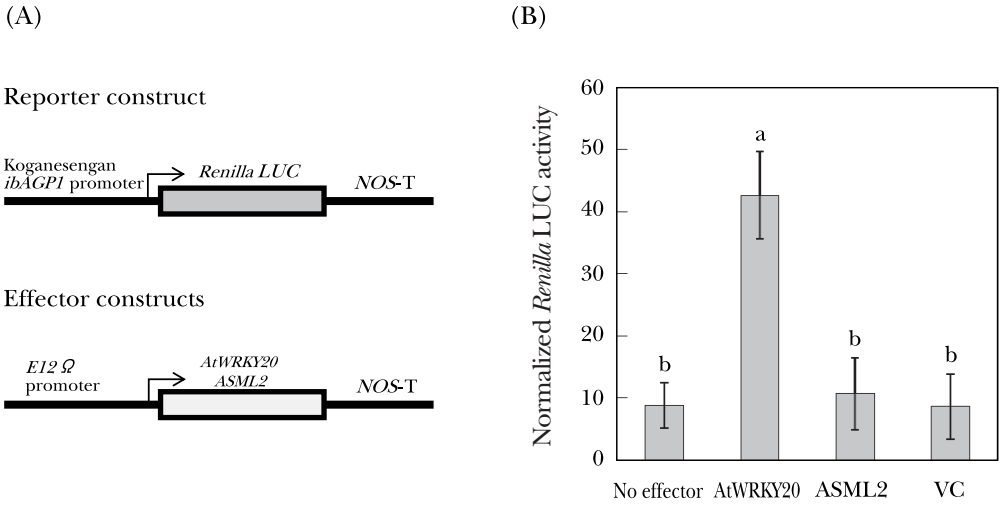

Fig. 4. Transient expression assays of the Koganesengan *ibAGP1* promoter by AtWRKY20 and ASML2 in sweet potato petioles. (A) Schematic representation of the reporter and effector constructs. The reporter construct contains a 1371-bp upstream sequence of the Koganesengan *ibAGP1* gene that controls *Renilla LUC* gene expression. In the effector constructs, expression of *AtWRKY20* and *ASML2* genes was driven by the *E12Ω* promoter. (B) Transient expression assays by co-bombardment of sweet potato petioles with the reporter and effector constructs and an internal control construct containing the firefly *LUC* gene. VC indicates the vector control. Values with the same letters do not differ significantly ($P < 0.05$, Tukey's test). Bars indicate SD (n=3).

the region between positions −640 and −180, and that this element is important for enhanced expression of the Koganesengan *ibAGP1* gene.

6. Binding of AtWRKY20 to the Koganesengan *ibAGP1* promoter

A DNA fragment that contained the Koganesengan *ibAGP1* promoter region from −623 to −426 was used as probe P1 (Fig. 6A). The P1 probe included one of the SURE-like fragments (SURE1) and two W-box core sequences (W-box1 and W-box2). In an electrophoretic mobility shift assay, addition of the AtWRKY20 protein efficiently retarded the mobility of the P1 probe, revealing two shift bands (Fig. 6B, lane 2). The P2 probe, which was a shorter version of the P1 probe without SURE1, bound to AtWRKY20, and only one shift band was detected (lane 4). Deletion of the SURE1 and W-box1 from the P1 probe (P3) effectively abolished the interactions (lane 6). On the other hand, with the P4 probe, which included SURE1 and W-box1, the assay detected two shift bands (lane 8). No retardation bands were detected with the P5 probe, which consisted of the Koganesengan *ibAGP1* promoter region from −373 to −157. These results indicate that AtWRKY20 interacts directly with the region between positions −623 and −490 in the Koganesengan *ibAGP1* promoter, but AtWRKY20 does not bind to W-box2, W-box3 and SURE2.

In conclusion, AtWRKY20 functions directly as a transcriptional activator of the *ApL3* promoter, and

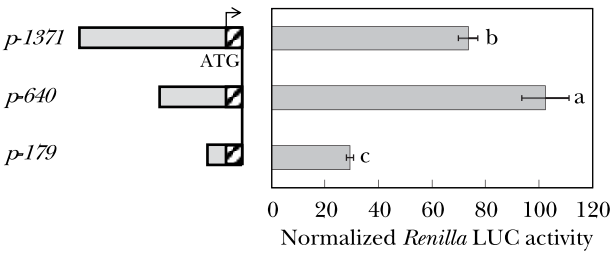

Fig. 5. Functional deletion analysis of the Koganesengan *ibAGP1* promoter. Chimeric constructs were obtained by fusion to the *Renilla LUC* gene (dashed box) of several Koganesengan *ibAGP1* promoter fragments whose nucleotide position, relative to the transcription initiation site, is indicated by the construct name. Petiole cross-sections were co-bombarded with the constructs and an internal control construct containing the firefly *LUC* gene. *Renilla* LUC activity was normalized relative to the firefly LUC activity for every independent transformation. Values with the same letters do not differ significantly ($P < 0.05$, Tukey's test). Bars indicate SD (n=3).

regulates the expression of *ApL3* induced by sucrose or osmoticum in *A. thaliana*. Moreover, AtWRKY20 can enhance the expression of the Koganesengan *ibAGP1* promoter directly in sweet potato. The *AtWRKY20* gene is a promising candidate to use in genetic engineering for improving sweet potato.

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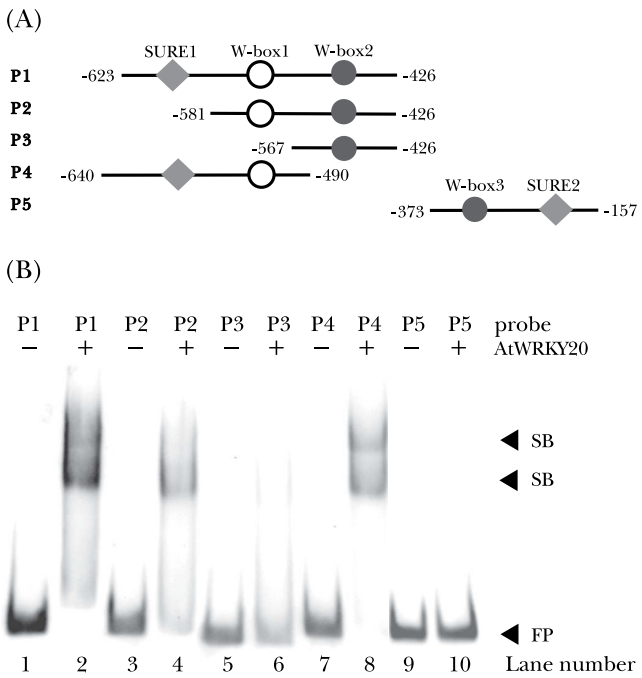

Fig. 6. Binding of AtWRKY20 to the Koganesengan *ibAGP1* promoter region. (A) Portions of the Koganesengan *ibAGP1* promoter used as probes. Diamonds denote SURE-like fragments, open circles represent W-box core sequences, and closed circles represent W-box core sequences with opposite orientation with respect to the direction of transcription. (B) Electrophoretic mobility shift assays of the Koganesengan *ibAGP1* promoter regions incubated with recombinant His-tagged AtWRKY20 protein. Arrowheads indicate the positions of the two shift bands (SB) and the free probe (FP).

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