Multiple transduction pathways regulate the 35S promoter with an ABA responsive element

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Abstract

A 53-bp DNA, ABRC1, with abscisic acid (ABA) responsive and coupling elements, was inserted into the 35S promoter controlling the expression of β -glucuronidase (GUS) gene, and its regulation on the transient expression of the GUS gene in tobacco protoplast was employed to examine the functions of ABA within plant cells. Two repeats of ABRC1 in its right orientation were ABA inducible in the 800-bp full-length and in the 90-bp minimal 35S promoters. However, neither methyl jasmonate nor salicylic acid induced the 35S promoter with ABRC1 elements. Applying okadaic acid, a protein phosphatase inhibitor, and staurosporine, a protein kinase inhibitor, revealed that the action of either dephosphorylated protein(s) or phosphorylated protein(s) enhanced GUS expression from the 35S promoter that contains ABRC1 elements. Nevertheless, the presence of both dephosphorylated protein(s) and other phosphorylated protein(s) may interfere in the transcription of 35S promoter alone. Conclusively, multiple pathways regulate the 35S promoter with the ABRC1 elements. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Abscisic acid; ABA responsive element; Okadaic acid; Staurosporine

1. Introduction

The plant hormone abscisic acid (ABA) importantly participates in plant growth and development such as seed development and the opening of stomata, and is involved in the adaptation to environmental stresses including temperature, salinity, and water [1]. Some ABA responses are rapid and affect ion fluxes, whereas others are long term and affect the regulation of gene expression. Many genes that respond to ABA have been isolated, and their corresponding proteins have been studied to provide details of their biological function [2]. The perception of ABA by cells initiates signal transduction pathways, including Ca^{+2} -independent events in

the cytoplasm, reversible protein phosphorylation, transcription factors, and several promoter *cis*-elements [3].

Several genes that were involved in the signal transduction of ABA have been characterized in *Arabidopsis* [4]. The *ABI1* and *ABI2* genes encode homologous protein phosphatase 2C [5], and *abi1* and *abi2* (ABA-insensitive) mutants exhibit abnormal stomata regulation and defects in various ABA-mediated responses to stress [1,6,7]. *Arabidopsis ABI3* gene, like the maize *VP1* gene, is involved in various aspects of seed development and germination, reflecting a reduced responsiveness to the hormone ABA [8]. Furthermore, using transient gene expression in rice protoplasts, maize *VP1* protein was shown to have additive and inhibitory effects with protein phosphatase, *ABI1-1*, from *Arabidopsis* [9]. Also, mutation in the *Eral* gene confers an enhanced response to exogenous ABA, and encodes the β -subunit of farnesyl transferase that modifies several signal transduction proteins for membrane localization [10]. *ABI5*, like *ABI1* and *ABI2* genes, was identified by selecting for seeds capable of germinating in the presence of an inhibitory ABA concentration [11], maintaining germinated embryos in a quiescent state

Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; CE, coupling element; ABRC1, ABA responsive complex 1; GUS, β -glucuronidase; MeJA, methyl jasmonate; SA, salicylic acid; STA, staurosporine; OKA, okadaic acid.

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to protect plants from drought [12]. These findings may reveal the multiple functions of ABA and the various signal pathways for ABA in regulating the expression of genes.

Promoter regions of genes induced by ABA always include an ABA-responsive element (ABRE), which functions as a *cis*-acting DNA element involving ABA regulation [13–15]. The 5' upstream sequence of the wheat *Em* promoter fused with β -glucuronidase (GUS) was first analyzed, and a 260-bp DNA fragment of the *Em* gene caused a 15-fold induction of GUS by ABA [16]. Further study indicated that a 75-bp fragment with ABRE from this *Em* gene inserted in either direction within the 35S promoter is responsible for ABA induction [17]. However, an ABRE sequence, ACGTGGC, resembles the G-box which is involved in a variety of environmental stimulations, such as UV light, white light, coumaric acid, and wounding [18,19]. A coupling DNA element (CE) in promoters other than G-box was proposed to specify the functions of the G-box [19]. G-box binding proteins, *trans* elements, were identified as leucine zipper proteins [18]. Also, three basic leucine zipper proteins, AREB1, AREB2, and AREB3, with ABRE-binding capability were isolated, and AREB1 and AREB2 function as transcriptional activators in the *Arabidopsis* gene *rd29B* [20]. The phosphorylation of AREB1 and AREB2 proteins is probably required for their maximum activation of ABA [20]. Therefore, the action of ABA in gene regulation involves the *cis* DNA elements and the *trans* transcription factors.

A 53-bp ABRE with CE from barley [19] was inserted in either direction in the 35S promoter, an ABA-insensitive promoter, and its participation in the presence of ABA can be analyzed in order to elucidate the functions of the *cis*-regulatory element during ABA activation. The responses of this ABRE to other stimuli, such as methyl jasmonate (MeJA) and salicylic acid (SA), were examined. Furthermore, modifications of the *trans*-regulatory factors involved in ABA signal transduction were investigated by applying inhibitors for protein kinases and phosphatases.

2. Materials and methods

2.1. Plant material and reagents

Nicotiana tabacum (L.) c.v. Wisconsin 38 was used in this study. Chemical reagents were purchased from Sigma (St. Louis, MO), and restriction and modify enzymes were obtained from Promega (Madison, WI).

2.2. Plasmid construction

The CaMV 35S promoter of pBI221SI [21] was digested into two parts. One 723-bp fragment, the 5'

Fig. 1. Schematic representation of the construction of plasmids 1R, 2R, 1L and 2L. The CaMV 35S promoter in pBI221SI [21] was divided into two parts. One 723-bp fragment, the 5' part of the 35S promoter, was isolated from the *Hind*III and *Eco*RV digestion of pBI221SI, and the 3' part 106-bp fragment, containing the CAT and TATA boxes of the 35S promoter, was isolated by the *Eco*RV and *Xba*I digestion from the pBI221SI vector. These two fragments from the 35S promoter were cloned into the corresponding sites of pBC SK+ to create pB35S. A unique *Eco*RI site between these two parts of the 35S promoter was maintained for the insertion of the ABRC1, which was constructed from four synthesized DNA oligonucleotides, LC1, LC2, LC3, and LC4 (Table 1). The *Hind*III–*Xba*I fragments (870–920 bp) of the pB35S derivatives, pB35S(ABRE), containing the 35S promoter and ABRC1 sequences, were subcloned back into the corresponding sites of pBI221SI to yield constructs 1R, 2R, 1L and 2L (Fig. 2) for electroporation assay. Constructs 1R and 2R contain one and two copies of the right orientation of ABRC1, and 1L and 2L contain one and two copies of the reversed orientation of ABRC1 in the –90 bp position of the full-length 800-bp 35S promoter.

part of the 35S promoter, was isolated by the *Hind*III and *Eco*RV digestion of pBI221SI. The downstream 106-bp fragment, containing CAT and TATA boxes of the 35S promoter, was isolated from *Eco*RV and *Xba*I digestion of the same vector (Fig. 1). The 723-bp *Hind*III–*Eco*RV fragment was cloned into the corresponding sites of pBC SK+ (Stratagene) to create vector pB5S, and the 106-bp *Eco*RV–*Xba*I fragment was constructed into the pB5S digested by *Sma*I and *Xba*I to produce vector pB35S (Fig. 1). A unique *Eco*RI site between these two parts of 35S promoter in pB35S was maintained for inserting ABRC1.

The ABA responsive complex 1, ABRC1 [19], was formed from four synthesized DNA oligonucleotides (Table 1). DNA oligonucleotides were treated by T4 polynucleotide kinase at 37 °C for 1 h in the presence of ATP. The mixture of four DNA oligonucleotides (10 μ M each), LC1, LC2, LC3, and LC4, was heated at 65 °C for 5 min, and kept overnight at 4 °C for ligation. The DNA oligonucleotide mixture after self-ligation was further ligated into the vector pB35S that was already digested by *Eco*RI and treated with calf intestinal

Table 1
DNA primers designed for construction

primer	sequence (5' to 3')
LC1	5'-AATTCCGGCTGCCCGCCACGTACAAGCACC-3' EcoRI ABRE3
LC2	5'-GCACCGGGTGCTTGCGGTGTACGTGGCGGGCAGCCGG-3' ABRE3
LC3	5'-CGGTGCCATTCGCCACCGG-3' CE1
LC4	5'-AATTCGGGTGGCAATG-3' EcoRI CE1
DEL1	5'-CCAAGCTTCCGCTGCCCCG-3' HindIII
DEL2	5'-GCTCTAGAGTCCCCCG-3' XbaI

alkaline phosphatase. The *Hind*III–*Xba*I fragment (870–920 bp) of the pB35S derivatives, containing 35S promoter and ABRC1, was further subcloned into the corresponding sites of pBI221SI [21] to produce constructs 1R, 2R, 1L and 2L for electroporation assay (Fig. 1). Constructs 1R and 2R contain one and two copies of the right orientation of ABRC1, and 1L and 2L contain one and two copies of the reversed orientation of ABRC1 in the –90 bp position of the full-length 800-bp 35S promoter (Fig. 2). The orientation of ABRC1 within the 35S promoter exhibits the right orientation (R), in which ABRE is located at the 5' position of the CE, and the reverse one (L), in which CE is located at the 5' position of the ABRE within ABRC1.

With DNA primers DEL1 and DEL2 (Table 1), the promoter regions of constructs D1R and D2R were generated from PCR reactions using constructs 1R and 2R as templates, respectively (Fig. 2). The PCR products were digested by *Hind*III and *Xba*I, and cloned into the corresponding sites of pBI221SI to generate constructs D1R and D2R, which contain the minimal 90-bp 35S promoter. All plasmids were identified by sequencing, and purified by CsCl–ethidium bromide isopycnic centrifugation [22] before electroporation.

2.3. Electroporation

The procedure for isolating protoplast from tobacco leaves (*Nicotiana tabacum*) has been described [23,24]. Plasmid MTC301 containing the 35S promoter and firefly luciferase was kindly provided by Dr. Su-May Yu (Institute of Molecular Biology, Academia Sinica, Taiwan), and was treated as an internal control during electroporation to compare quantitatively the GUS expression from different constructs. The electroporation procedure was reported [21], and modified as

Fig. 2. Major components of the constructs used in this study. The CaMV 35S promoter in pBI221SI [21] is an 800-bp promoter including TATA box (TATA). Only a part of the GUS gene is shown. The procedure for constructing plasmids 1R, 2R, 1L, and 2L is described in Fig. 1. The insertion of ABRC1 within the –90 bp region, the *Eco*RV site, of the 35S promoter is represented as a dark arrow. The orientation of ABRC1 within the 35S promoter is the right orientation (R), in which ABRE is located at the 5' position of the CE element, and represented as a dark arrow that points to the right. The reverse orientation (L), in which the CE element is located at the 5' position of the ABRE within ABRC1, is shown as a dark arrow that points to the left. The promoter regions of constructs D1R and D2R were generated from PCR reactions using constructs 1R and 2R as templates, respectively. The PCR products were digested by *Hind*III and *Xba*I, and cloned into the corresponding sites of pBI221SI to produce constructs D1R and D2R, which contain the minimal 90-bp 35S promoter with TATA box.

follows. Protoplast isolated from tobacco leaves was adjusted by a dilution solution (0.4 M D-mannitol, 8 mM MES–KOH, and 5 mM CaCl₂) to obtain 2×10^6 cell/ml. The mixture that contained 8 µg testing DNA constructs, 8 µg plasmid MTC301, 10 µg calf thymus DNA, and 50 µl PEG solution (24% w/v polyethylene glycol 4000, 0.4 M D-mannitol, 30 mM MgCl₂, pH 5.6) was added to 100 µl protoplast following heat shock. Electroporation was performed by Baekon 2000 electroporator (Baekon, Inc, Saratoga, CA) with conditions recommended by the manufacturer (2KV, 22 pulse, 0.4 s burst time, five cycles, and 62.5 µs). Following centrifugation at $800 \times g$ for 5 min, protoplast pellets were resuspended in an incubation solution (MS salt, 1% sucrose, and 0.4 M D-mannitol) at 25 °C for 16 h.

2.4. GUS assay

The procedure for GUS assay was described by Jefferson [25], and was modified as follows. After incubation with DNA for 16 h, protoplast was collected by centrifugation at $800 \times g$ for 5 min, and was resuspended in a 75 µl extraction buffer (100 mM potassium phosphate buffer (pH 7.7), 1 mM EDTA, 8 mM 2-mercaptoethanol, 0.5% Triton X-100, and 10% glycerol). Cells were broken down by three cycles of

alternate freezing and thawing, and the supernatant of the cells was collected by centrifugation at $10\,000 \times g$ for 5 min. The supernatant (50 μ l) was incubated with 150 μ l of 4-MUG assay buffer at 37 °C [25]; 45 μ l of this reaction mixture was on each occasion taken out at 0, 10, 20, and 40 min by adding 1 ml of 0.2 M sodium carbonate. The fluorescence of the samples was measured by the excitation wavelength at 365 nm and the emission wavelength at 455 nm, using a Hitachi Spectrofluorometer F2000. A solution of 100 mM 4-methyl umbelliferone (MU) in 0.2 M sodium carbonate was used to calibrate the intensity of fluorescence.

2.5. Luciferase assay

A method for the assay of firefly luciferase has been described [26]. Luciferase activity was measured using the Optocomp 1 Bacterial System (GEM Biomedical Inc.).

2.6. Protein assay

The concentration of the protein in each cell extract was determined using the Coomassie Blue Binding method [27].

2.7. Data calculation

The GUS assays of each sample involve four instances in time. The slope with the units of pmol MU/min can be obtained from the linear regression through these four points [25]. This GUS result was adjusted by the protein concentration for the amount of cells in each sample, and by the luciferase activity for the efficiency of electroporation. Final adjusted data in units of pmol MU/min/mg protein were presented in this study. All results were repeated at least six times, and given as mean $\pm$ standard deviation. The expression value of construct 1R in the absence of ABA was treated as the normalized reference, with a value of one, to determine the relative GUS activities of other constructs.

3. Results

In terms of transient assay, the expression of a reporter is influenced by the efficiency of the electroporation process. For quantitative analysis in particular, a second reporter gene is needed as an internal reference of electroporation for the comparison among samples. The luciferase gene in this study was used as the second reporter in all assays, and was required to obtain the repeatable results (data not shown). The concentration of protein normalized the quantity of protoplast used among samples, and this control eliminated the deviation in the analyses due to the differences in the cell

masses in each sample. In order to compare the transient expression of GUS among experiments, the GUS expression of construct 1R in the absence of ABA was always performed in every set of assays, and treated as the normalized reference, with a value of one, to determine of the relative GUS expression.

3.1. Functions of ABRC1 in 35S promoter

Promoters with ABRE and CE were activated by the addition of ABA in barley [19]. The functioning of a similar ABRE-like sequence in other ABA-insensitive promoters like the 35S promoter was examined. The ABRC1 [19] assembled from oligonucleotides LC1, LC2, LC3, and LC4 (Table 1) was self-ligated to form monomer and dimer ABRC1, and then constructed into the full-length 800-bp 35S promoter (Fig. 1). The orientation of ABRC1 within the 35S promoter shows the right orientation (R), in which ABRE is located at the 5' position of the CE element, and the reverse orientation (L), in which CE is located at the 5' position of the ABRE within ABRC1. ABRC1 was located at the –90 region of the 35S promoter in plasmid pBI221SI [21]. One copy of ABRC1 with the right (1R) and reversed (1L) orientations, and two copies with the right (2R) and reversed (2L) orientations were obtained (Fig. 2). These constructs were electroporated into tobacco protoplast in the absence and presence of 0.1, 1, or 10 μ M ABA. The GUS expression of construct 2R in 1 μ M ABA was dramatically greater than that of other constructs in the presence or absence of ABA (Fig. 3). Compared to the 35S promoter without ABRC1 in pBI221SI (C in Fig. 6B), the inserting ABRC1 reduced the activity of 35S promoter (C in Fig. 6A). However, the 35S promoter with ABRC1 elements became ABA-inducible with ABRC1 element. This may indicate that the full-length 800-bp 35S promoter, into which two copies of the right orientation of ABRC1 from barley have been inserted, is inducible by ABA within tobacco protoplast, although the induction of constructs 1R, 1L, and 2L by ABA is insignificant (Fig. 3). The 53-bp ABRC1 element is responsible for the induction of ABA since the 35S promoter alone is not induced by ABA (C and A in Fig. 6B).

3.2. Functions of ABRC1 in the minimal 35S promoter

Using constructs 1R and 2R as templates, PCR reactions were performed with DNA primers DEL1 and DEL2 (Table 1) to produce constructs D1R and D2R, respectively (Fig. 2). Constructs D1R and D2R contain the ABRC1 sequence and the minimal 35S promoter, which is a 90-bp element containing TATA box, and the effect of ABA on these constructs was studied by the transient expression of GUS. Construct D2R with two right copies of ABRC1 can be induced by

Fig. 3. Effects of ABA concentration on constructs 1R, 2R, 1L, and 2L. Fig. 2 illustrates the structures of constructs 1R, 2R, 1L, and 2L. Eight micrograms of constructs 1R, 2R, 1L, or 2L with GUS reporter and 8 μ g plasmid MTC301 with luciferase gene as an internal control were electroporated into tobacco protoplasts in the absence and presence of 0.1 to 10 μ M ABA. After 16-h incubation, the GUS activity, the luciferase activity, and the protein concentration of the cell extract were analyzed (Section 2). The value of the GUS activity was adjusted according to its corresponding luciferase activity and protein concentration for equality of electroporation and cell mass, respectively. After these adjustments, the expression of construct 1R without ABA was treated as the normalized reference, with a value of one, to determine the relative GUS activities of other constructs. Data are presented as the mean $\pm$ standard deviation from at least six independent experiments.

0.1 and 1 μ M ABA, while 0.1 μ M ABA slightly enhanced the GUS expression of D1R, which contains one right copy of ABRC1 (Fig. 4). However, the GUS expression of construct 1R in the absence of ABA was also treated as a reference with a value of one in all assays of this study. Therefore the quantity of GUS expression from D2R with the minimal 35S promoter was ten times lower than that of construct 2R with the full-length 800-bp promoter (Figs. 3 and 4). Nevertheless, the minimal 35S promoter alone did not respond to ABA (data not shown); ABRC1 in minimal 35S promoter is inducible by ABA.

3.3. Effects of methyl jasmonate acid and salicylic acid on the 35S promoter with ABRC1

MeJA like ABA was involved in the plant defense system [28], and SA induced the synthesis of pathogen-related proteins [29]. The protoplast-containing construct 2R with two right copies of ABRC1 was treated with MeJA and SA to examine whether these compounds participated in inducing the expression of the 35S promoter with ABRC1. The relative GUS activity remained roughly unchanged when the concentration of MeJA was 5 μ M, and gradually declined to zero when

Fig. 4. Effects of ABA concentration on constructs D1R and D2R. Fig. 2 displays the structures of constructs D1R and D2R. Eight micrograms of constructs D1R or D2R with GUS reporter and 8 μ g plasmid MTC301 with luciferase gene as an internal control were electroporated into tobacco protoplasts in the absence and presence of 0.1 to 10 μ M ABA. After 16-h incubation, the GUS activity, the luciferase activity, and the protein concentration of cell extract were analyzed (Section 2). The value of GUS activity was adjusted by its corresponding luciferase activity and the protein concentration for equality of electroporation and cell mass, respectively. Following such adjustments, the expression value of construct 1R in the absence of ABA, data not presented in this figure, was treated as the normalized reference, with a value of one, to determine the relative GUS activities of other constructs. Data are presented as the mean $\pm$ standard deviation from at least six independent experiments.

the concentration of MeJA was from 50 to 5000 μ M (Fig. 5A). The GUS activity of construct 2R in the presence of SA at 0.01 mM was about the same as that in the absence of SA (Fig. 5B). When the concentration of SA varied from 0.1 to 10 mM, the expression of GUS is progressively reduced to zero (Fig. 5B). These results demonstrate that neither MeJA nor SA can induce the 35S promoter with ABRC1 elements.

3.4. Effects of okadaic acid and staurosporine on the 35S promoter with ABRC1

Phosphorylation and dephosphorylation of proteins play an important role in signal transduction in plants [30,31]. Okadaic acid (OKA) and staurosporine (STA) inhibit protein phosphatase 1/2A and kinase, respectively [32,33], and have been widely employed to study the action of protein phosphatase and kinase in cells. When either OKA or STA was applied to protoplast, the expression level of GUS protein from construct 2R during transient expression was similar to that stimulated by ABA alone (O, S, and A in Fig. 6A). However, construct pBI1221SI containing only the 35S promoter without ABRC1 did not enhance its GUS expression, after treatment with either OKA or STA (O and S in

Fig. 5. Effects of MeJA and SA on construct 2R. The structure of construct 2R is indicated in Fig. 2. Eight micrograms of constructs 2R with GUS reporter and 8 μg plasmid MTC301 with luciferase gene as internal control were electroporated into tobacco protoplasts in the presence of MeJA (A) or SA (B) as indicated. Following 16-h incubation, the GUS activity, the luciferase activity, and the protein concentration of cell extract were analyzed (Section 2). The value of GUS activity was adjusted by its corresponding luciferase activity and the protein concentration for equality of electroporation and cell mass, respectively. After such adjustments, the expression value of construct 1R in the absence of ABA, data not presented in this figure, was treated as the normalized reference, with a value of one, to determine the relative GUS activities of other constructs. Data are presented as the mean $\pm$ standard deviation from at least six independent experiments.

Fig. 6B). Therefore, ABRC1 element was responsible for GUS induction during the addition of OKA and STA. This finding may also indicate that the action of dephosphorylated protein(s) or phosphorylated pro-

Fig. 6. Effects of ABA, STA, and OKA on constructs 2R and pBI221SI. (A) Effects on construct 2R. Fig. 2 shows the structure of construct 2R that contains two copies of ABRC1. (B) Effects on pBI221SI. Fig. 2 gives the structure of pBI221SI. The 35S promoter within pBI221SI is an 800-bp full-length promoter without ABRC1. Eight micrograms of construct 2R or pBI221SI with GUS reporter and 8 μg plasmid MTC301 with luciferase gene as internal control were electroporated into tobacco protoplasts in the presence of 1 μM ABA, 0.1 μM OKA, or/and 0.1 μM STA. The treatments of protoplast with ABA, OKA, or STA are indicated as A, O, S, respectively. The simultaneous co-treatments of protoplast with OKA and STA, ABA and OKA, ABA and STA, or ABA, OKA, and STA are represented as OS, AO, AS, and AOS, respectively. The protoplast without any treatment was also performed as a control (C). After 16-h incubation, the GUS activity, the luciferase activity, and the protein concentration of the cell extract were analyzed (Section 2). The value of GUS activity was adjusted according to its corresponding luciferase activity and protein concentration to maintain electroporation and cell mass, respectively. After such adjustments, the expression value of construct 1R in the absence of ABA, data not presented in this figure, was treated as the normalized reference, with a value of one, to determine the relative GUS activities of other constructs. Data are presented as the mean $\pm$ standard deviation from at least six independent experiments.

tein(s) leads to the enhancement of GUS expression from construct 2R that contains ABRC1 elements.

However, when both OKA and STA were added simultaneously, the GUS expression from construct 2R was dramatically decreased in either the absence or presence of ABA (OS and AOS in Fig. 6A). A similar reduction in GUS expression due to the presence of both OKA and STA was also observed in pBI221SI (OS and AOS in Fig. 6B). This inhibition may therefore result from the 35S promoter rather than the ABRC1 element. Since the functions of OKA and STA are to inhibit protein phosphatase and kinase, respectively, the presence of both dephosphorylated protein(s) and other phosphorylated protein(s) may interfere with the basic transcription of 35S promoter alone.

When both ABA and OKA were applied to the protoplast with construct 2R (AO in Fig. 6A), the enhanced level of GUS expression was similar to that due to treatment with either ABA or OKA alone (A and O in Fig. 6A). Also, the target element in promoter for both ABA and OKA is the same, the ABRC1 element, and therefore the induction of GUS protein by ABA may occur through phosphorylated protein(s) (Fig. 7). The action of OKA prevents the phosphorylated proteins from dephosphorylation, and the function of ABA may stimulate the production of phosphorylated proteins. However, the GUS expression of construct 2R when treated with ABA and STA (AS in Fig. 6A) was lower than that when with either ABA (A in Fig. 6A) or STA (S in Fig. 6A). STA inhibits protein kinase, preventing the dephosphorylated proteins from phos-

phorylating. Also, ABA may stimulate the production of phosphorylated protein(s), as mentioned above. Consequently, the presence of both dephosphorylated protein(s) due to the addition of STA and other phosphorylated protein(s) stimulated by ABA may interfere in the basic transcription machinery of the 35S promoter, as described above (OS in Fig. 6). However, ABA may stimulate the production of phosphorylated proteins more than that of dephosphorylated proteins caused by STA; the excess phosphorylated proteins can still activate GUS expression in construct 2R. Hence, the expression of GUS protein from construct 2R in the presence of both ABA and STA is between that in the presence of OKA and STA (OS in Fig. 6A) and that in the presence of ABA (A in Fig. 6A). This finding may reveal the involvement of multiple transduction pathways in activating the ABRC1 element within the 35S promoter (Fig. 7).

4. Discussion

The sequence of ABRC1, a 53-bp DNA, was synthesized according to the promoter region of the barley HVA22 gene [34]. ABRC1 contains a G box-like sequence ABRE, GCCACGTACA, and a coupling sequence CE, TGCCACCGG, and was reportedly sufficient for ABA induction [19]. During the transient expression of barley seeds by particle bombardment, the tandem repeat of four copies ABRC1 connected to Amy64 minimal promoter activates more than 100-fold of ABA induction in reporter GUS expression [19]. Here, significant induction by ABA was observed only when two copies of the right orientation of ABRC1 were inserted into the 35S promoter (Figs. 3 and 4). Since the leucine zipper-type ABRE-binding proteins were isolated, the interaction between ABRE-binding proteins and ABRE *cis* elements could be the primary cause for the ABA induction of transcription [20]. Hence, the requirement for the multiple copies of ABRE elements in ABA induction is probably due to the synergistic effect from the *cis* DNA elements bound by the *trans* transcription factors.

The 53-bp ABRC1 element is responsible for ABA induction in both the full-length and minimal 35S promoter (Figs. 3 and 4). However, the maximum induction level of ABA in the full-length 35S promoter with ABRC1 element is about seven fold (2R), while that in the minimal 90-bp 35S promoter is about four fold (D2R). The 5' end of the full-length 35S promoter, which is absent from the 35S minimal promoter, seems to enhance ABA induction, indicating perhaps that regulatory factors binding this 5' end DNA sequence of the full-length 35S promoter assist the ABRC1 element in ABA induction.

Fig. 7. A model of the ABA signal transduction for regulating the ABRC1 element within the 35S promoter. ABA may activate protein kinase, and then increase the production of phosphorylated proteins. While OKA suppresses the activity of protein phosphatase 1/2A, preventing phosphorylated proteins from being converted to dephosphorylated proteins (P1s). Therefore, the action of either ABA or OKA results in the activation of ABRC1 within the 35S promoter. STA inhibits the activity of protein kinase, and prevents the dephosphorylated proteins (P2s) from phosphorylating. STA also activates the ABRC1 within the 35S promoter. However, the co-existence of phosphorylated proteins and dephosphorylated proteins interferes in the basic transcription of 35S promoter alone. Arrows indicate positive action, while blunted lines show negative regulation.

MeJA has been considered to be an essential signal molecule during the activation of defense-related proteins [35]. Both ABA and MeJA can activate genes of *WAPK* in tobacco, cyclophilins in potato, and leucine aminopeptidase in tomato [31,36,37]. These genes are co-regulated by ABA and MeJA. However, an MeJA-inducible protein of sweet potato, ipomoelin, acts independently of ABA [38]; MeJA induces the expression of a chitinase from *Brassica juncea* but is unaffected by ABA [39]. The ABRC1 element within 35S promoter was identified to be ABA-responsive, but to be unaffected by MeJA (Fig. 5). These findings may indicate that genes are differently regulated by ABA and MeJA.

SA induces the synthesis of pathogen-related proteins and enhances plants to resist a number of pathogens [29]. SA, however, was demonstrated to inhibit the accumulation of MeJA and the subsequent gene expression [40]. Treatment of leaves detached from *S. chacoense* with ABA or SA resulted in moderate accumulation of DS2 mRNA [41], but an ABA-inducible acid phosphatase (AtACP5) from *Arabidopsis thaliana* was not regulated by SA [42]. The ABRC1 element within the 35S promoter was inducible by ABA, but was not affected by SA (Fig. 5). Again, genes were differently affected by ABA and SA.

Signal transduction induced by ABA was studied, and several protein factors involved in ABA regulation were identified. *ABI1* and *ABI2* are essential components in ABA signal transduction, and encode protein phosphatase 2C [5]. However, adding OKA, a protein phosphatase inhibitor, to the protoplast enhances the activity of the promoter with the *cis*-element ABRE in construct 2R (Fig. 6), and may not interfere in the action of *ABI1* and *ABI2*, which are also protein phosphatases. This result could be due to the inhibition specificity of OKA is protein phosphatase 1 and 2A rather than protein phosphatase 2C. Also, the signal transduction pathways conducted by *ABI1* and *ABI2* may differ from that or those inhibited by OKA. In addition, the leucine zipper-type proteins, AREB1, AREB2, and AREB3, were isolated, and identified as ABRE *cis*-element binding proteins. The phosphorylation of these AREB proteins has been suggested to be important for their activation [20]. Consequently, the inhibition of OKA may prevent the phosphorylated AREB proteins from dephosphorylating, resulting in the activation of the ABA-inducible promoter.

ABA is importantly involved in the adaptation to environmental stress and in seed maturation and dormancy. It regulates the expression of different genes through various signal transduction pathways. As mentioned above, *ABI1* and *ABI2* genes encode protein phosphatase [5], and phosphorylation of AREB regulatory proteins is involved in activating ABA [20].

Reversible phosphorylation of proteins is clearly the important process in the ABA transduction pathway. When the protoplast with construct 2R was treated with ABA and OKA, the increasing level of GUS expression was similar to that when the construct was treated with either ABA or OKA alone (Fig. 6A). Because ABA alone, OKA alone, or both do not enhance the GUS expression of 35 promoter by itself (Fig. 6B), the ABRC1 element in construct 2R is the target element for the action of ABA and OKA. Therefore, the induction of GUS protein by ABA may occur through phosphorylated protein(s), and ABA and OKA may participate in the same reversible phosphorylation of protein(s) (Fig. 7). While the action of OKA prevents the phosphorylated protein(s) from dephosphorylation, the function of ABA may promote the production of phosphorylated protein(s). Thus, the action of ABA alone, OKA alone, or both together activates the ABRC1 element in construct 2R, and enhances the expression of GUS (Fig. 6A).

The addition of STA, a protein kinase inhibitor, alone to protoplast also increases the expression of GUS in construct 2R (S in Fig. 6A), and negligibly alters GUS expression from the background 35S promoter in pBI221SI (S in Fig. 6B). Dephosphorylated protein(s) also seems to be involved in the activation of ABRC1, and results in the increasing GUS expression. However, the simultaneous addition of both STA and OKA to the tobacco protoplast greatly reduces the GUS expression from not only construct 2R but also from pBI221SI with or without the presence of ABA (Fig. 6A and B). The mechanism of the basic transcription from 35S promoter alone is likely to be suppressed in the presence of both protein kinase(s) and phosphatase(s) inhibitors simultaneously (Fig. 7). Surprisingly, when STA was applied with ABA to tobacco protoplast, its GUS expression was lower than that treated with either ABA or STA alone (Fig. 6A). This result may highlight that the dephosphorylated protein(s) presented because of the function of STA and other phosphorylated protein(s) produced by the action of ABA can individually activate ABRC1 in the construct 2R. The presence of both dephosphorylated and phosphorylated proteins due to the simultaneous action of STA and ABA may, however, interfere with the transcription of the 35S promoter by itself. Nevertheless, the GUS expression produced by STA together with ABA treatment still exceeds that by simultaneous STA and OKA treatment (Fig. 6A). This implies that while ABA enhances the production of phosphorylated protein(s) which co-react with dephosphorylated protein(s) to reduce 35S promoter activity, ABA can still stimulate ABRC1 activity in a manner unaffected by STA. These results also reveal that the protein kinase inhibited by STA is not involved in the ABA signal transduction (Fig. 7).

Apparently, multiple transduction pathways are involved in regulating ABRC1, an ABA-inducible element. The interactions among protein factors in regulating transcription are complicated.

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