Plant Cell Physiol. 43(1): 136–140 (2002)

Short Communication

Experimentally Determined Sequence Requirement of ACGT-Containing Abscisic Acid Response Element

Tsukaho Hattori [1], Masako Totsuka, Tokunori Hobo, Yasuaki Kagaya and Akiko Yamamoto-Toyoda

Center for Molecular Biology and Genetics, Mie University, Tsu, 514-8507 Japan

The sequence requirement of the ACGT-containing abscisic acid response element (ABRE) was analyzed by systematically substituting the bases surrounding the ACGT-core of motif A, the principal ABRE of the rice gene, *Osem.* This was done within the context of a 55-bp promoter fragment that minimally confers ABA-responsiveness to a heterologous promoter. Based on this analysis, the sequence requirement of the ACGT-containing ABRE was determined as ACGTG G/T C, which matched very well with the consensus derived from sequence comparison of ABA-responsive promoters.

Key words: ABA — ABRE — bZIP protein — Rice (*Oryza sativa*) — Stress.

Abbreviations: ABREs, ABA-responsive elements; bZIP, basic region-leucine zipper; FI, fold-induction; IL, induced level; PCR, polymerase chain reaction; GUS, β-glucuronidase.

Abscisic acid (ABA) is involved in various physiological and developmental processes including stress responses and seed maturation (Giraudat et al. 1994). During these processes, the level of endogenous ABA is increased, and thereby various genes are induced (Giraudat et al. 1994, Busk and Pages 1998). *Cis*-elements called ABA response elements (ABREs), which primarily mediate ABA-induced transcription, have been identified by functional analyses and sequence comparison of ABA-inducible promoters (Busk and Pages 1998, Giraudat et al. 1994, Guiltinan et al. 1990, Hattori et al. 1995, Hobo et al. 1999a, Marcotte et al. 1989, Mundy et al. 1990, Pla et al. 1993, Skriver et al. 1991, Thomas et al. 1997). Since multimerized ABREs can confer ABA-responsiveness to a heterologous, minimal promoter (Skriver et al. 1991, Vasil et al. 1995), this sequence intrinsically possesses the capacity to mediate ABA signals. In a natural promoter context, an ABRE functions with a second sequence element called the "coupling element" (Shen and Ho 1995, Shen et al. 1996). The two elements together constitute an ABA responsive *cis*-element complex (ABRC), which can synergistically activate transcription in response to ABA. Two distinct coupling elements, namely CE1 (Shen and Ho 1995) and CE3 (Shen et al. 1996) have been identified. Subsequently, we demonstrated that the CE3 sequence functions as a second copy of the ABRE in the rice *Osem* promoter (Hobo et al. 1999a).

ABREs are typically recognized by the presence of the ACGT-core (Hobo et al. 1999a, Marcotte et al. 1989, Mundy et al. 1990, Pla et al. 1993). However, recent studies have indicated that some ABREs, including the CE3 sequence of the *Osem* promoter, contain GCGT or AAGT instead of ACGT (Ezcurra et al. 2000, Ono et al. 1996, Hobo et al. 1999a). We have shown that these ACGT-containing and non-ACGT ABREs are *cis*-elements of essentially the same nature and are recognized by the same transcription factor(s) (Hobo et al. 1999a, Hobo et al. 1999b). In the case of non-ACGT ABREs, more limited sequences appear to be allowed on both sides of the core (Hobo et al. 1999a).

Following a literature search in addition to experiments in our laboratory, we have proposed a sequence of $T/G/C_{-2}$ $A_{-1}C_0G_0T_{+1}G_{+2}$ G/T_{+3} C_{+4} [following the nucleotide numbering by Izawa et al. (1993)] as the consensus for ACGT-containing ABREs (Hattori and Hobo 1999, Hobo et al. 1999a). However, putative ABRE sequences have varied between laboratories because the sequence requirement for ABRE has not been critically discussed before. For a more accurate prediction of ABREs, it would be helpful to experimentally determine the sequence requirement of the element by systematically mutating each base of a functional ABRE. However, as the sequence requirement of ABREs may be influenced by the context of flanking sequences and other *cis*-elements that function in combination, it may not be absolutely defined. Nevertheless, it is doubtlessly beneficial to experimentally determine the base specificity of each position of the ABRE sequence in order to predict functional ABREs and study their interactions with ABRE binding factors (Choi et al. 2000, Finkelstein and Lynch 2000, Hobo et al. 1999b, Uno et al. 2000).

We previously defined a minimal fragment of the rice *Osem* promoter that is able to confer ABA-responsiveness to the CaMV 35S (–46) minimal promoter (Hobo et al. 1999a). This fragment of 55 bp contains an ACGT-containing ABRE (motif A) and a CE3 sequence (non-ACGT ABRE). Both the motif A and CE3 sequences act synergistically, resulting in a high level of ABA induction, and are interchangeable (Hobo et

[1] Corresponding author: E-mail, hattori@gene.mie-u.ac.jp; Fax, +81-59-231-9048.

Table 1 5′ mutant primers used for PCR amplification

Mutation	5′ Sequence 3′
A_{-2}	CCAAGCTTGCGCACG*A*ACGTGTC
C_{-2}	CCAAGCTTGCGCACG*C*ACGTGTC
G_{-2}	CCAAGCTTGCGCACG*G*ACGTGTC
A_{+2}	CCAAGCTTGCGCACGTACGT*A*TCGC
C_{+2}	CCAAGCTTGCGCACGTACGT*C*TCGC
T_{+2}	CCAAGCTTGCGCACGTACGT*T*TCGC
A_{+3}	CCAAGCTTGCGCACGTACGTG*A*CGC
C_{+3}	CCAAGCTTGCGCACGTACGTG*C*CGC
G_{+3}	CCAAGCTTGCGCACGTACGTG*G*CGC
A_{+4}	CCAAGCTTGCGCACGTACGTGT*A*GC
G_{+4}	CCAAGCTTGCGCACGTACGTGT*G*GC
T_{+4}	CCAAGCTTGCGCACGTACGTGT*T*GC
$G_{-4}A_{-3}C_{-2}$	CCAAGCTTGCGCA*GA*CACGTGTC
$G_{-4}C_{-3}C_{-2}$	CCAAGCTTGCGCA*GCC*ACGTGTC
$C_{+2}G_{+3}$	CCAAGCTTGCGCACGTACGT*CG*CGC
$G_{-4}C_{-3}G_{-2}/C_{+2}G_{+3}$	CCAAGCTTGCGCA*GCG*ACGT*CG*CGC

Mutated bases are shown in italics

al. 1999a). In the present study, we examined the sequence requirement of the ACGT-containing ABRE by making every possible substitution of bases flanking the ACGT core of motif A of the *Osem* promoter.

Base substitutions were systematically created by polymerase chain reaction (PCR) using a 5′ mutant primer with various nucleotide substitutions and an 8 bp extension for a *Hin*dIII restriction site (Table 1) and a 3′ common primer (5′-TGCGAAGATCTCGAGCCTCC-3′). A 55-bp-TATA plasmid, containing a chimeric promoter consisting of the 55-bp *Osem* promoter fragment and the CaMV 35S (–46) minimal promoter, in addition to the β-glucuronidase (GUS) coding region and the nopaline synthase terminator (Hobo et al. 1999a; Fig. 1) was used as a template. The same plasmid was used for the backbone plasmid to replace the *Osem* promoter fragment with the amplified mutant promoter fragment. The ABRE mutants created in this way were assayed in a transient expression system using cultured rice cell protoplasts (Hattori et al. 1995, Hobo et al. 1999a).

In the experiments described here, the wild-type 55-bp-TATA exhibited a 25- to 89-fold induction of GUS expression by ABA. Although the level of ABA induction with the wild-type construct varied from one experiment to another, the fluctuations among independent electroporations with the same protoplast preparation were reasonably small, as previously reported (Hattori et al. 1995). Therefore, the values were compared only within an experiment with the same protoplast preparation. Since mutations of the ABRE affected not only the fold-induction (FI) value but also the induced level of expression (IL), the effect of a mutation was evaluated for both parameters. In the previous experiments, extensive base substitution of motif A in the 55-bp context still exhibited a 4- to 6-fold induction of GUS expression, which corresponded to approximately 10–15% of the wild-type value (Hobo et al. 1999a). Thus, this level of reduction in FI by a mutation is considered to be essentially a total loss of ABRE function.

In "Experiment 1" (Fig. 2 exp. 1), the base requirement at the –2 position was examined. Any substitution at this position did not significantly affect the FI value. The IL was decreased to 49 and 56% of the wild-type value when T_{-2} was substituted with A and G, respectively. Substitution with C also resulted in a slight decrease in IL. This result indicates that the –2 position is not critical to the ABRE function although T or C is preferred.

Any substitution of G at the +2 position resulted in large decreases in both the FI and IL as revealed by "Experiment 2" (Fig. 2 exp. 2). The moderate FI of the A_{+2} mutant relative to the wild-type value (26%) was partly due to the comparatively low FI of the wild-type construct in this particular experiment. Judging from the resulting low IL values of any mutation of the G at the +2 position, it is considered to be essential to ABRE function.

In "Experiment 3" (Fig. 2 exp. 3), a substitution of T_{+3} with A resulted essentially in a complete loss of ABRE function judging from both the FI and IL values. The C_{+3} mutant also exhibited a very low FI value. The increase in IL (19% of the wild-type value) appeared to be due to the increase in the basal level of expression, which may be caused by binding of a transcription factor other than ABRE-binding factors. In contrast to the C_{+3} and A_{+3} mutants, substitution with G_{+3} resulted in only a slight reduction of FI. These results indicate that the +3 position requires T or G for ABRE function.

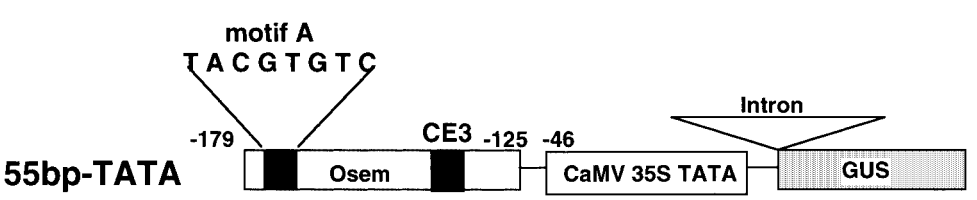
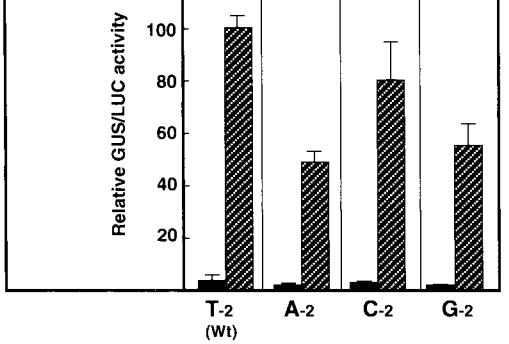


Fig. 1 Schematic illustration of the 55-bp-TATA construct used to create the base-substitution mutants of the ABRE. The 55-bp promoter fragment was inserted into pIG46 (Ono et al. 1996) containing the CaMV 35S minimal promoter, the modified castor bean CAT1 intron and the GUS coding region. The 55-bp fragment contains motif A (an ACGT-containing ABRE) and the CE3 element (non-ACGT ABRE). The italicized bases in motif A were systematically mutated and tested for ABRE function.

Exp. 1

-4 -3 -2 -1 0 0 +1 +2 +3 +4
C G *T-2*A C G T G T C

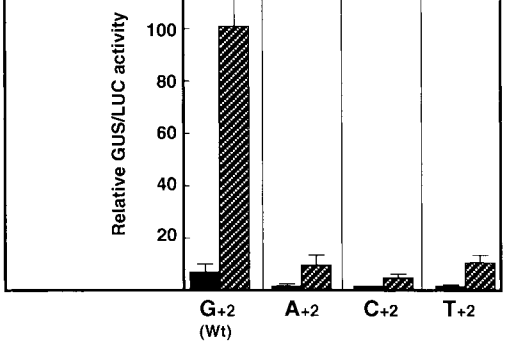
Fold-induction (FI)	28x (100%)	28x (100%)	31x (110%)	33x (118%)
Induced level (IL)	100	49	80	56



Exp. 2

-4 -3 -2 -1 0 0 +1 +2 +3 +4
C G T A C G T *G+2*T C

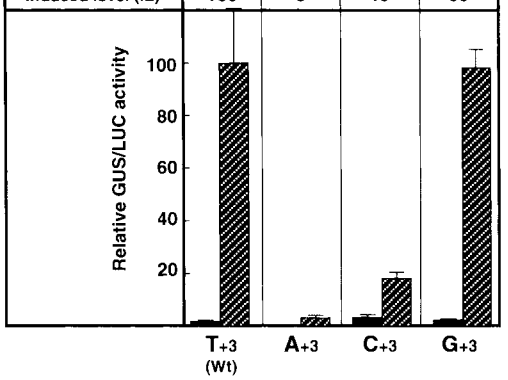
Fold-induction (FI)	25x (100%)	7x (26%)	3x (12%)	5x (20%)
Induced level (IL)	100	11	3	8



Exp. 3

-4 -3 -2 -1 0 0 +1 +2 +3 +4
C G T A C G T G *T+3*C

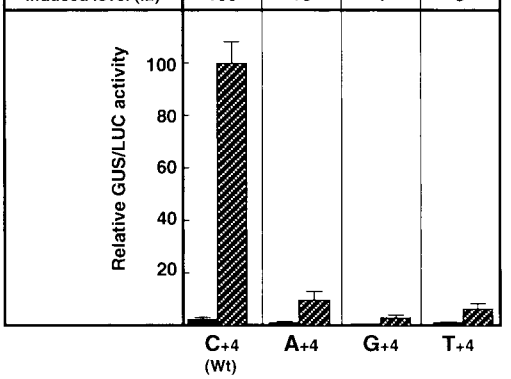
Fold-induction (FI)	51x (100%)	5x (10%)	7x (14%)	42x (82%)
Induced level (IL)	100	3	19	96



Exp. 4

-4 -3 -2 -1 0 0 +1 +2 +3 +4
C G T A C G T G T *C+4*

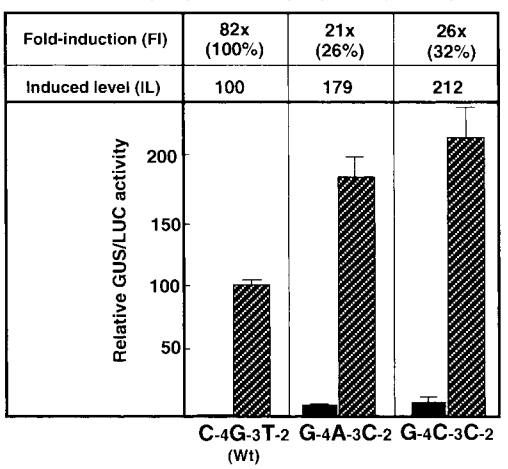
Fold-induction (FI)	38x (100%)	15x (39%)	8x (21%)	9x (23%)
Induced level (IL)	100	10	4	8



Exp. 5

-4 -3 -2 -1 0 0 +1 +2 +3 +4
*C-4G-3T-2*A C G T G T C

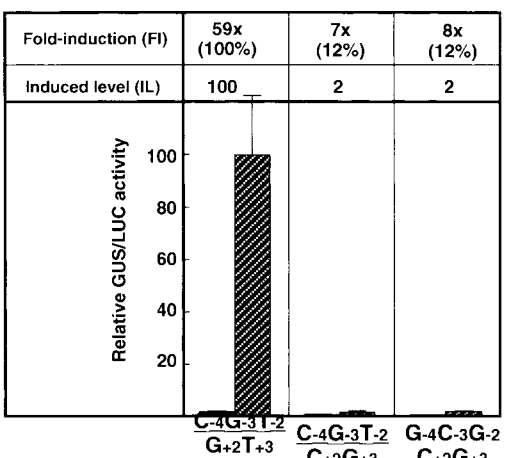
Fold-induction (FI)	82x (100%)	21x (26%)	26x (32%)
Induced level (IL)	100	179	212



Exp. 6

-4 -3 -2 -1 0 0 +1 +2 +3 +4
*C-4G-3T-2*A C G T *G+2T+2*C

Fold-induction (FI)	59x (100%)	7x (12%)	8x (12%)
Induced level (IL)	100	2	2



Mutations at the +4 position also greatly affected the ABRE function (Fig. 2 exp. 4). The IL value of any base substitution at this position resulted in a significant reduction that was 10% or less of the wild-type value. FI values were also greatly reduced by any mutations at this position. Thus, C is strongly preferred at the +4 position. However, the relative FI value of the A_{+4} mutant (39% of the wild-type value) was significantly higher than the FI values of the other mutants. Therefore, the sequence including A_{+4} may function as a weak ABRE.

Based on the above experiments, the sequence requirement of the ABRE for the bases flanking ACGT is summarized as $A_{-1}C_0G_0T_{+1}G_{+2}$ G/T_{+3} C_{+4}. This experimentally derived ABRE sequence almost completely matches with the above-mentioned consensus derived from sequence comparison. In addition, our results essentially coincide with the recognition sequences of an Arabidopsis ABRE binding factor ABF1 as determined by a binding site selection experiment (Choi et al. 2000). Since the sequence was not defined by testing all possible combinations of base substitutions or selecting functional sequences from random sequences, it should be cautioned that it may not be an absolute requirement. However, the good agreement with the consensus, as well as the recognition sequence of the ABRE-binding factor, supports our conclusion.

An ABRE of the wheat *Em* promoter, Em1a (GCACACGTGGC; Marcotte et al. 1989) is a dyad of the 3′ 5 bp ($G_0T_{+1}G_{+2}$ G/T_{+3} C_{+4}) of the ABRE sequence defined by the above experiments. In contrast, other ACGT-containing ABREs such as motif A (TACGTGTC) and motif I (TACGTGGC; Mundy et al. 1990) of *Rab16A* are not dyads except for the ACGT core. We have previously shown that the ABRE binding factor TRAB1 binds with greater affinity to the dyad ABRE than to the non-dyad ABREs (Hobo et al. 1999b). In "Experiment 5" (Fig. 2 exp. 5), we created either an Em1a sequence or a complete dyad (GACACGTGTC) in place of motif A in the 55-bp context and then tested for ABRE function. Both constructs gave approximately a twofold higher IL compared to the wild type. However, these increases in IL accompanied more pronounced elevation of basal expression, which resulted in a significant reduction of the FI. The dyad composed of the GTG G/T C half site is known to be a strong binding sequence for various "group I" bZIP proteins (see below; Izawa et al. 1993), which include those apparently unrelated to ABA-regulation. The high level of basal expression with the dyad constructs may have been driven by such bZIP factors. Since the nature of basal expression in transient assay systems is not known, the low FI observed with the dyad constructs does not necessarily indicate its poor function as an ABRE. As expression of stress-related ABA-responsive genes is generally suppressed to very low levels under the uninduced conditions, endogenous chromosomal genes appear to be equipped with some mechanism that ensures the suppression of basal expression. Therefore, the dyad sequences may be able to serve as an ABRE that can give higher levels of expression when induced. However, the twofold difference in the ILs between the dyad and non-dyad ABREs is not large, compared to the difference in the binding affinities of TRAB1, and probably of other ABRE-binding factors, to them. Therefore, the affinities to the non-dyad ABREs in vivo appear to be sufficient for the ABRE function.

The central 6-bp, CACGTG, of the dyad G A/C CACGTG G/T C is referred to as the G-box. Similarly, GACGTC is defined as the C-box. Izawa et al. (1993) classified plant b-ZIP proteins into three groups according to their binding sequence specificities. While "group I" and "group III" bZIP proteins preferentially bind to dyads containing a G-box and a C-box, respectively, "Group II" bZIP proteins bind to dyads containing either a G-box or a C-box (Izawa et al. 1993). As shown in "Experiment 6" (Fig. 2 exp. 6), a C-box containing dyad, GCGACGTCGC, which is a preferred binding site of "group II" bZIP proteins, exhibited only 2% and 12% of the wild-type values of IL and FI, respectively. Therefore, the C-box dyads for "group II" bZIP protein binding do not function as ABREs. Similarly, the motif A derivative, in which the 3′ half site was replaced with the half site of the C-box dyad, did not function well either.

The sequence requirement of the ABRE experimentally defined here and the consensus sequence for the ABRE derived from sequence comparison matched very well. However, the significance of the two is clearly distinct. While the consensus sequence only can predict the sequences that may function as an ABRE, our results have clarified those sequences which are similar to functional ABREs but do not function or are unfavorable as ABREs. We believe that our results may be used as a more reliable guide to predict ABREs or the responsiveness of a given promoter to ABA. For a more comprehensive understanding of the sequence requirement of ABREs, it will be important to extend similar studies to non-ACGT type ABREs.

Fig. 2 Effect of base substitution surrounding the ACGT-core of motif A on ABRE function. In each experiment, the wild-type 55-bp-TATA construct, or the mutant derivative with the indicated base-substitution, was electroporated together with an ubiquitin::luciferase construct as an internal standard into rice cell protoplasts and cultured in the presence (hatched bars) or absence (black bars) of ABA. GUS and luciferase activities were assayed after 40 h of culture. Values of GUS activities normalized to luciferase activities are relative to those obtained with the wild-type 55-bp-TATA in the presence of ABA, and are the means of four independent electroporations with standard errors. Each experiment was conducted with a single protoplast preparation. Levels of expression relative to the wild type under the induced condition (IL), and ABA-induction expressed by fold-induction (FI), are indicated on top of each pair of bars.

Acknowledgments

This work was funded in part by Grants-in-Aid for Scientific Research on Priority Areas (A) (Grant 12037208) and (B) (Grant 12138204) from the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT).

References

Busk, K. and Pages, M. (1998) Regulation of abscisic acid-induced transcription. *Plant Mol. Biol.* 37: 425–435.

Choi, H., Hong, J., Ha, J., Kang, J. and Kim, S.Y. (2000) ABFs, a family of ABA-responsive element binding factors. *J. Biol. Chem.* 275: 1723–1730.

Ezcurra, I., Wycliffe, P., Nehlin, L., Ellerstrom, M. and Rask, L. (2000) Transactivation of the Brassica napus napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different cis-elements: B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G-box. *Plant J.* 24: 57–66.

Finkelstein, R.R. and Lynch, T.J. (2000) The Arabidopsis abscisic acid response gene *ABI5* encodes a basic leucine zipper transcription factor. *Plant Cell* 12: 599–609.

Giraudat, J., Parcy, F., Bertauche, N., Gosti, F., Leung, J., Morris, P.C., Bouvier-Durand, M. and Vartanian, N. (1994) Current advances in abscisic acid action and signalling. *Plant Mol. Biol.* 26: 1557–1577.

Guiltinan, M.J., Marcotte, W.R. and Quatrano, R.S. (1990) A plant leucine zipper protein that recognizes an abscisic acid response element. *Science* 250: 267–270.

Hattori, T. and Hobo, T. (1999) Regulation of gene expression by abscisic acid in rice. *In* Molecular Biology of Rice. Edited by Shimamoto, K. pp. 139–160. Springer-Verlag, Tokyo.

Hattori, T., Terada, T. and Hamasuna, S. (1995) Regulation of the *Osem* gene by abscisic acid and the transcriptional activator VP1: analysis of cis-acting promoter elements required for regulation by abscisic acid and VP1. *Plant J.* 7: 913–925.

Hobo, T., Asada, M., Kowyama, Y. and Hattori, T. (1999a) ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. *Plant J.* 19: 679–689.

Hobo, T., Kowyama, Y. and Hattori, T. (1999b) A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. *Proc. Natl. Acad. Sci. USA* 96: 15348–15353.

Izawa, T., Foster, R. and Chua, N.-H. (1993) Plant b-ZIP protein DNA-binding specificity. *J. Mol. Biol.* 230: 1131–1144.

Marcotte, W.R. Jr, Russel, S.H. and Quatrano, R.S. (1989) Abscisic acid-responsive sequences from the Em gene of wheat. *Plant Cell* 1: 969–976.

Mundy, J., Yamaguchi-Shinozaki, K. and Chua, N.-H. (1990) Nuclear proteins bind conserved elements in the abscisic acid-responsive promoter of a rice rab gene. *Proc. Natl. Acad. Sci. USA* 87: 1406–1410.

Ono, A., Izawa, T., Chua, N.-H. and Shimamoto, K. (1996) The rab16B promoter of rice contains two distinct abscisic acid-responsive elements. *Plant Physiol.* 112: 483–491.

Pla, M., Vilardell, J., Guiltinan, M.J., Marcotte, W.R., Niogret, M.F., Quatrano, R.S. and Pages, M. (1993) The *cis*-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maize gene *rab28*. *Plant Mol. Biol.* 21: 259–266.

Shen, Q. and Ho, T.-H.D. (1995) Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and novel cis-acting element. *Plant Cell* 7: 295–307.

Shen, Q., Zhang, P. and Ho, T.-H.D. (1996) Modular nature of abscisic acid (ABA) response complexes: composite promoter units that are necessary and sufficient for ABA induction of gene expression in barley. *Plant Cell* 8: 1107–1119.

Skriver, K., Olsen, F.L., Roger, J. and Mundy, J. (1991) Cis-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. *Proc. Natl. Acad. Sci. USA* 88: 7266–7270.

Thomas, T.L., Chung, H.J. and Nunberg, A.N. (1997) ABA signaling in plant development and growth. *In* Signal Transduction in Plants. Edited by Aducci, P. Birkhauser Verlag, Basel.

Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc. Natl. Acad. Sci. USA* 97: 11632–11637.

Vasil, V., Marcotte, W.R. Jr, Rosenkrans, L., Cocciolone, S.M., Vasil, I.K., Quatrano, R.S. and McCarty, D.R. (1995) Overlap of Viviparous1 (VP1) and abscisic acid response elements in the *Em* promoter: G-box elements are sufficient but not necessary for VP1 transactivation. *Plant Cell* 7: 1511–1518.

Yamaguchi-Shinozaki, K., Mundy, J. and Chua, N.-H. (1989) Four tightly linked *rab* genes are differentially expressed in rice. *Plant Mol. Biol.* 14: 29–39.

(Received September 17, 2001; Accepted October 31, 2001)