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Regulation by External and Developmental Signals of the *RAV2* Gene Encoding a DNA Binding Protein Containing AP2 and B3-Like Domains

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Abstract

Arabidopsis RAV1 and RAV2 are unique transcription factors that contain two distinct DNA binding domains, namely AP2 and B3-like domains, both of which have been identified only in higher plants. We characterized the DNA binding properties and expression of RAV2. RAV2 was shown to bind to the same sequences recognized by RAV1. Expression of *RAV2* mRNA was found to be modulated by mechanical stimuli. Developmental regulation of *RAV2* was investigated using transgenic plants carrying a *RAV2* promoter-GUS construct. The analyses revealed complex developmental regulation of *RAV2* expression. In particular, the spatial pattern of GUS expression in roots suggested a possible function of RAV2 related to lateral root formation.

1. Introduction

Most plant transcription factors fall into families, each of which share a DNA binding domain conserved among eukaryotes (Meshi and Iwabuchi, 1995). Zinc finger, basic-leucine zipper (bZIP), basic-helix-loop-helix (bHLH), MADS box, and homeo-box are well-known examples of conserved DNA binding domains. However, some DNA binding domains, such as B3-(like) domain and AP2-(like) domain (Weigel, 1995 and Okamuro *et al.*, 1997) have been identified only in higher plants. The AP2 domain was first identified as a DNA-binding domain conserved in a family of tobacco ethylene response element binding proteins (EREBPs) (Ohme-Takagi and Shinshi, 1995), and later found to be conserved in *Arabidopsis* APETALA2 (AP2) (Jofuku *et al.*, 1994) and other proteins (Klucher *et al.*, 1996; Elliott *et al.*, 1996; Wilson *et al.*, 1996; Stockinger *et al.*, 1997 and Finkelstein *et al.*, 1998). The B3 domains have been identified as a highly conserved domain in VP1/ABI3 proteins, which are involved in abscisic acid (ABA)-regulated gene expression during seed maturation (McCarty, 1995). A sequence-specific DNA binding activity of the B3 domain has recently been reported (Suzuki *et al.*, 1997). In addition to VP1/ABI3 proteins, B3-like domains are found in *Arabidopsis* ARF1 (Ulmasov *et al.*, 1997) and FUS3 (Luerssen *et al.*, 1998). We have recently cloned cDNAs for unique DNA binding proteins, designated RAV1 and RAV2, which contain both AP2 and B3-like domains (Kagaya *et al.*, 1999). Binding site selection assays and other biochemical characterization of recombinant RAV1 proteins have revealed that RAV1 binds to bipartite recognition sequences consisting of two unrelated sequence motifs, CAACA and CACCTG, which are recognized by the AP2 and B3-like domains, respectively (Kagaya *et al.*, 1999). A striking feature of RAV1 is that it flexibly recognizes bipartite target sequences, in which the two motifs are separated by various spacings in two different relative orientations. The amino acid sequence of RAV2 is 67% identical to that of RAV1. However, the sequence identities of the AP2 and B3-like domains between the two proteins are as high as 81% and 82%, respectively. Properties of RAV2 as to DNA binding and expression have not previously been investigated. In addition, the physiological functions of RAV1 and RAV2 are not known because RAV proteins were identified based on sequence information available at the expression sequence tag (EST) databases. Here we have investigated the DNA binding properties of RAV2 and its gene expression to obtain a clue to its physiological function.

2. Materials and Methods

2.1 Plant growth conditions and treatments

Arabidopsis thaliana (ecotype, Columbia) plants

were grown on agar media containing MS salts (Murashige and Skoog, 1962) and 1% sucrose at 22°C under continuous light. For plant transfer treatment, 4-day-old seedlings were removed from the media and placed on a 10 cm petri dish containing 20 ml distilled water. The seedlings were then taken out of the petri dish after a certain time period and frozen immediately with liquid N_2. For wounding treatment, leaves and stems of 14-day-old plants were cut into pieces with a razor blade and floated on distilled water. After a certain time period, tissues were immediately frozen as above.

2.2 RNA extraction and gel blot hybridization analysis

Extraction of RNA from frozen tissues and RNA gel blot hybridization were carried out as described previously (Yamaguchi-Shinozaki and Shinozaki, 1994).

2.3 Electrophoretic mobility shift assays (EMSAs)

An expression plasmid for the production of glutathione S-transferase (GST) fusion protein with the entire region of RAV2 (GRAV2-ent) was constructed by the same methods described for GRAV1-ent (Kagaya *et al.*, 1999). Preparation of probes, binding reaction, and electrophoresis for EMSAs were carried out as described previously (Kagaya *et al.*, 1999).

2.4 Plant transformation

A Ti-binary vector plasmid containing a *RAV2* promoter-GUS chimeric gene was constructed as follows. A genomic clone containing the *RAV2* gene was isolated from an *Arabidopsis thalian* (ecotype, Landsberg *erecta*) λ-*DASH*II genomic library. A 4.5 kb *Bam*HI/*Hin*dIII fragment containing a 0.3 kb coding region (proximal to the *Bam*HI site) and a 4.2 kb 5′ franking region was subcloned into pBluescript. From the pBluescript clone, a 1.8 kb fragment of the *RAV2* promoter ending at the initiation ATG was prepared by unidirectional deletion with exonuclease III and mungbean nuclease, and PCR-amplification. Then the promoter fragment was once inserted upstream of the β-glucuronidase (GUS) coding region of pBI221 (Jefferson, 1987) in place of the CaMV 35S promoter. A fragment containing the *RAV2* promoter, GUS coding region and NOS terminator was cut out from the pBI221 clone and used to replace the 35S-Ac fragment of pB-Ac101 (Greveldding *et al.*, 1992). The resultant plasmid was used for plant transformation via *Agrobacterium tumefacience* (GV3101). The vacuum infiltration method (Bechtold *et al.*,1993) was used to transform *Arabidopsis thaliana* (ecotype, Columbia) plants.

2.5 Histochemical staining of GUS activity

Plant tissues were fixed with 90% (v/v) cold acetone for 10 min, rinsed with 50 mM sodium phosphate buffer (pH7.0) and stained for GUS activity with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) as described previously (Jefferson *et al.*, 1986).

3. Results

3.1 RAV2 binds to the same bipartite sequences recognized by RAV1

Since RAV2 possesses both AP2 and B3-like domains highly homologous to those of RAV1, we examined whether RAV2 binds to the same sequences as those recognized by RAV1. Recombinant RAV2 protein produced in *E. coli* as a GST fusion protein (GRAV2-ent) was used for EMSAs. As shown in **Fig. 1**, GRAV2-ent fusion protein formed DNA-protein complexes with a typical recognition sequence (sequence 6-1; see ref. Kagaya *et al.*, 1999) of RAV1

WT	TCGACGGCAACAATAAACACCTGACTCGA
mACA	TCGACGGCAtgtATAAACACCTGACTCGA
mCCT	TCGACGGCAACAATAAACAataGACTCGA
mACA/CCT	TCGACGGCAtgtATAAACAataGACTCGA

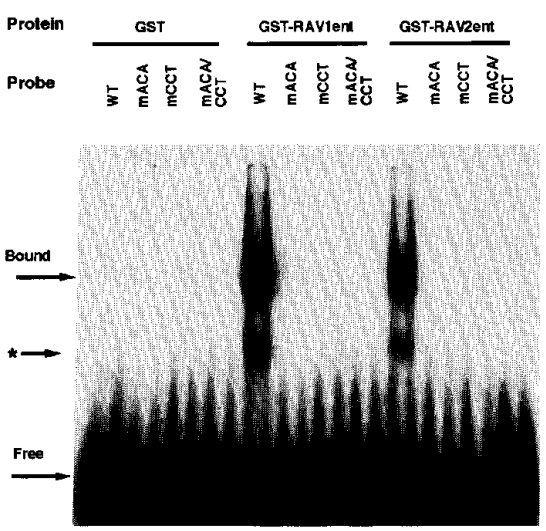

Fig. 1 EMSAs showing that RAV2 binds to the same sequence recognized by RAV1.
A lysate (2 μg protein) of *E. coli* expressing GST alone, GRAV1-ent or GRAV2-ent was used for binding reaction as indicated. The sequences of probes used are listed at the top. WT is a typical RAV1 binding sequence (6-1; see ref. Kagaya *et al.*, 1999). mACA, mCCT and mACA/CCT are mutant probes, in which the AACA, CACCTG and both motifs, respectively, are mutated. The arrow with an asterisk indicates the complexes with proteolytic degradation products.

but not with its mutant sequences, in which the CAACA, CACCTG or both motifs were mutated. No DNA-protein complexes with similar mobilities were observed when a lysate of *E. coli* cells that expressed only GST was used. We also confirmed the binding of RAV2 to other RAV1 recognition sequences. These results indicate that RAV2 can bind to the bipartite RAV1 target sequence by recognizing the CAACA and CACCTG motifs as RAV1 does.

3.2 *Distribution of RAV2 mRNA among organs*

Distribution of *RAV2* mRNA among organs was analyzed by RNA gel blot hybridization. **Fig. 2** shows that *RAV2* mRNA was present at relatively high levels in roots and rosette leaves and at low levels in cauline leaves and siliques at 5-10 days after flowering. Although the autoradiogram in **Fig. 2** gave no clear signals for *RAV2* mRNA in inflorescent stems and flowers, we were able to confirm the signals by a radioisotope imaging instrument (BAS-1000; Fuji Film, Tokyo) (data not shown). The results indicate that the distribution of *RAV2* mRNA was similar to that previously reported for *RAV1* mRNA although *RAV2* mRNA was detected in inflorescent stems and flowers at very low levels. The size of *RAV2* mRNA was approximately 1.4 kb except for that present in siliques, which was smaller by 0.2 kb. The reason for the smaller size transcripts is not known.

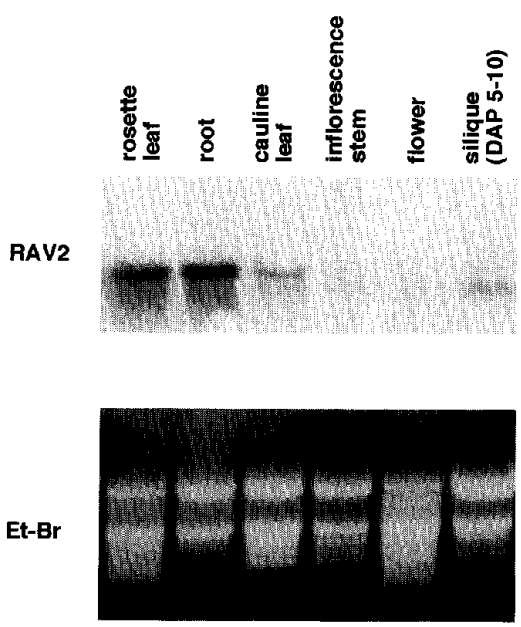

Fig. 2 Distribution of *RAV2* mRNA among organs examined by RNA gel blot hybridization analysis.
Ten micrograms of total RNAs prepared from indicated organs of *Arabidopsis* plants were size fractionated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with ^{32}P-labeled RAV2 cDNA. The photograph of ethidium bromide-stained agarose gel before RNA transfer is shown at the bottom.

3.3 *Alterations in RAV2 expression by mechanical stimuli*

Since RAV1 and RAV2 contain a B3-like domain which exhibits homology to the B3 domains of ABI3/VP1 proteins, we examined the possibility of regulation of the expression of *RAV2* by ABA. However, we instead found that the control treatment increased the level of *RAV2* mRNA (data not shown). This observation led us to examine the time course of change in the level of *RAV2* mRNA after simply transferring plants to distilled water. As shown in **Fig. 3-A**, the level of *RAV2* mRNA transiently decreased at 1 h after transfer and then increased. At 3 h it was already higher than the original level and reached a maximum at 9 h. The most likely cause of the change in *RAV2* expression appeared to be some kind of mechanical stimuli such as touch. A change in pH or osmolarity could be another possibility. We also examined the effect of wounding as a different mechanical stress. As shown in **Fig. 3-B**, the level of *RAV2* mRNA decreased at 3 h after wound treatment, but did not change thereafter. We observed a decrease in the level of *RAV2* mRNA as early as at 1 h (data not shown). Although the real nature of stimuli generated by the tested treatment is not clear in both cases, the expression of *RAV2* is subject to dynamic change in response to external stimuli.

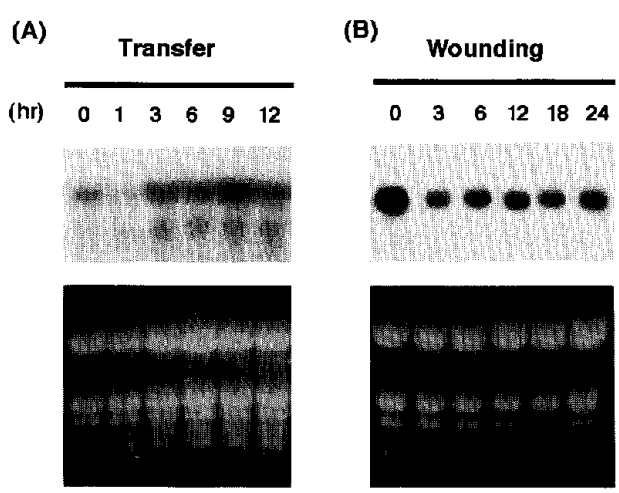

Fig. 3 RNA gel blot hybridization analysis showing that transferring plants (A) or wounding (B) alters the levels of *RAV2* mRNA.
Total RNAs from the tissues harvested at the indicated times after the treatment were subjected to RNA gel blot hybridization analysis as in **Fig. 2**. Four-day-old seedlings and 14-day-old plants were used for the experiments in A and B, respectively.

3.4 Histochemical analysis of transgenic plants carrying a RAV2 promoter-GUS fusion gene

Although cases are known, where the expression of a promoter-GUS chimeric gene does not faithfully reflect the expression pattern of the original gene, analyses with promoter-GUS transgenic plants are still a powerful and convenient way to study the expression of a given gene if results are interpreted with care. Thus, we carried out histochemical analysis of GUS expression with transgenic *Arabidopsis* plants that carried a chimeric gene (RAV2-GUS), in which a GUS coding region was placed downstream of a 1.8 kb *RAV2* promoter fragment.

Very young seedlings (3 day-old) exhibited GUS staining in the root (**Fig. 4-A**). A lower GUS activity was also observed in the hypocotyl. The staining in both root and hypocotyl was restricted to regions close to the hypocotyl-root junction. In the root, GUS activity was observed at and around the vascular tissue. In the hypocotyl, both epidermal and inner tissues exhibited GUS activity. In the cotyledons, GUS activity was observed only at their tips. At this stage, the vascular system of cotyledons was not yet well developed. No GUS staining was observed in the true leaf primordia or shoot apical meristem.

In the 7-day-old seedlings (**Fig. 4-B**), when the cotyledons were fully expanded, but the first true leaf had not yet developed, the upper part of the hypocotyl tissues including the vascular system exhibited GUS activity. At this stage, a remarkable GUS activity emerged in the cotyledons. The spatial pattern of GUS activity in the cotyledons was somewhat unusual. Numerous spots of GUS staining with a size of several cells were observed with a uniform but fainter background staining. A clear staining was also observed in the vascular tissues of cotyledons. The roots also exhibited an interesting GUS expression pattern. The GUS activity was observed only in the vascular tissues with alternating regions exhibiting high and low GUS activities.

In adult plants (14 to 28-day-old), high levels of GUS activity were observed in the roots (**Fig. 4-C**). The expression was again confined to vascular tissue. A closer examination of the root tissues, the activity was found in the cells inner pericycle cell layer, but not in the epidermal, cortical and endodermal layers (**Fig. 4-D**). The alternating pattern of strong and weak expression regions was more prominent than that observed in the young seedlings. Very interestingly, the regions with higher GUS activities were centered where a lateral root primodium was present (**Fig. 4-C** and **D**). The lateral root priomodia themselves were also stained strongly. In rosette leaves (**Fig. 4-E**), a spotty pattern of staining similar to that observed in the cotyledons of the 7-day-old seedlings was again observed, in addition to the staining in the vascular tissues. In the cauline leaves (**Fig. 4-F**), the expression was often observed at their tip and the vascular veins of the basal region. The stem-cauline leaf and stem-secondary stem junctions (**Fig. 4-G**) were also stained. In flowers (**Fig. 4-H**), the anthers and pollen grains were strongly stained for GUS activity. In immature siliques (**Fig. 4-I**), the vascular tissues and developing seeds exhibited high levels of GUS activity. GUS stain was also observed in the developing embryos (data not shown).

The high levels of GUS expression in the roots and rosette leaves of transgenic plants were in agreement with the results of RNA gel blot hybridization analysis. However, the strong GUS staining in the anthers did not appear to be reflected in the hybridization analysis. A possible explanation for this apparent discrepancy is that RNA in anthers was not readily extractable when whole flowers were used. Alternatively, the information contained in the 1.8 kb *RAV2* promoter could be insufficient to exert the strict regulation exhibited by the endogenous gene.

4. Discussion

We have shown here that RAV2 can bind to the same sequence that is recognized by RAV1. In addition, the organ distribution of *RAV2* mRNA is similar, though not identical, to that of *RAV1* mRNA (high levels in roots and leaves and low in other organs) (Kagaya *et al.*, 1999). These results indicate that the two DNA binding proteins may be involved in the regulation of the same or overlapping sets of target genes. Although we have not examined the tissue specificity and developmental regulation of the *RAV1* expression, the two genes might function in different tissues. Alternatively, the function of the two genes could be simply redundant. Another possibility is that they may compete with each other for the same target sites acting as antagonizing effectors such as an activator and repressor. Studies with transgenic plants carrying *RAV1* promoter-GUS and *in situ* hybridization analyses will provide a clue to these questions.

The expression of *RAV2* was found to be under the regulation of both external stimuli and internal developmental signals. It appears difficult to picture a single common physiological process, where RAV2 functions, at different stages and tissues, from the complex expression patterns observed in the present study. It would rather be likely that RAV2 is involved in the regulation of several distinct physiological processes.

Although other possibilities are not excluded, one likely cause of the change in *RAV2* expression upon

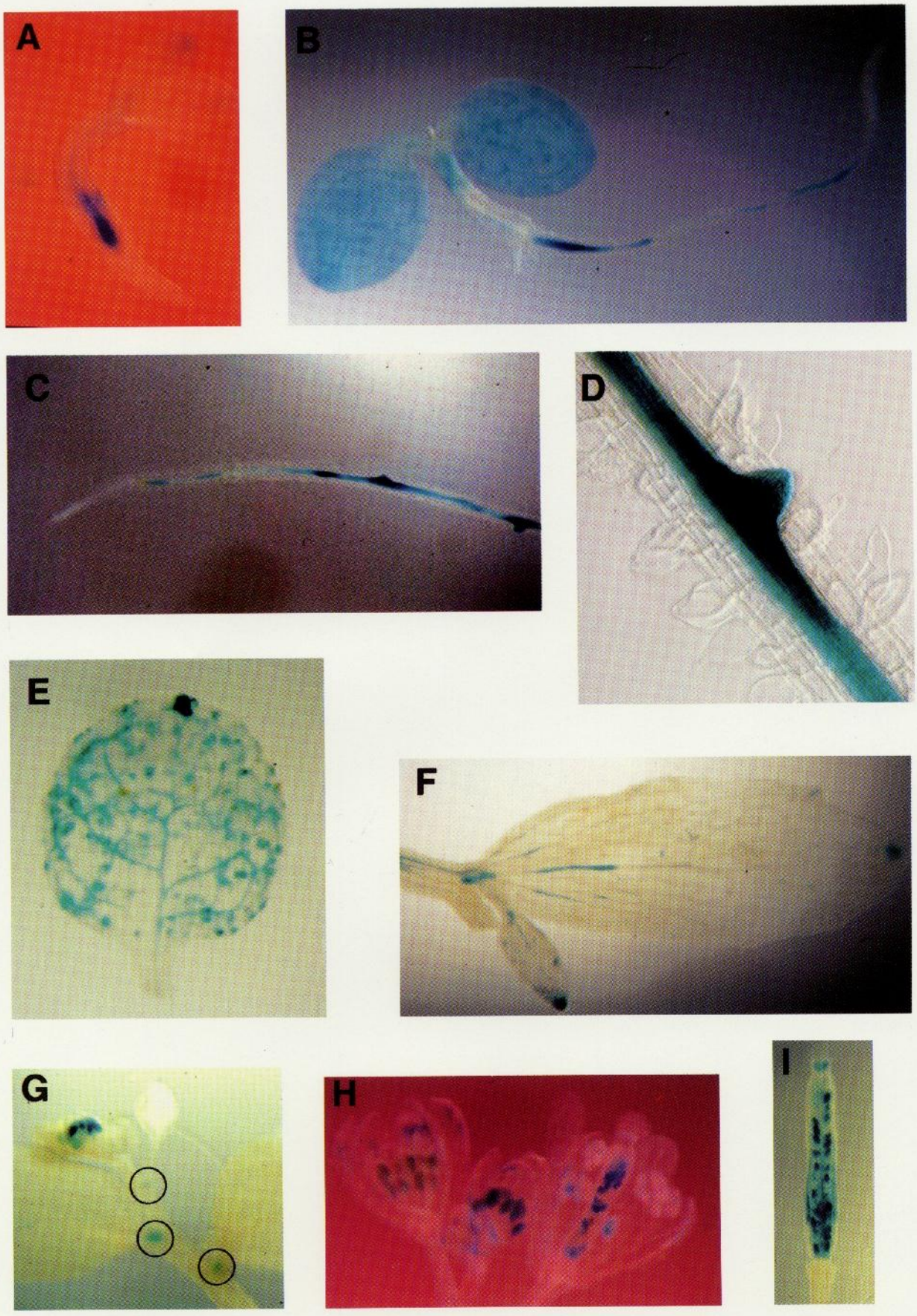

Fig. 4 Histochemical GUS staining of transgenic *Arabidopsis* plants carrying the RAV2-GUS fusion gene.
(A) 3-day-old seedling; (B) 7-day-old seedling; (C) root of 14-day-old plants; (D) the same as (C) with a higher magnification; (E) rosette leaf of 14-day-old plant; (F) cauline leaf of 20-day-old plant; (G) stem-cauline leaf and stem-secondary stem junctions (encircled); (H) flowers; (I) immature silique.

transfer of plants is mechanical stimuli as in the case of the touch-induced genes. Touch-induced genes have been shown to be expressed in the regions where plants experience mechanical stresses during normal development. For example, touch genes such as *TCH3* (Sistrunk *et al.*, 1994) and *TCH4* (Xu *et al.*, 1995) are expressed at the junctions where a cauline leaf or secondary stem is attached. The GUS expression at the stem-secondary stem and stem-cauline leaf junctions in the RAV2-GUS transgenic plants is consistent with the hypothesis that *RAV2* is regulated by touch or similar stimuli. Wounding treatment is inevitably accompanied by touch stimuli. Thus, the wound-induced down-regulation of *RAV2* expression and the transient decrease in *RAV2* expression upon transfer of plants could be a common phenomenon, but in wounded tissues, the subsequent up-regulation process might be inhibited.

The most remarkable observation with RAV2-GUS transgenic plants was the spatial pattern of GUS expression in roots. Strong GUS staining was observed in the regions centered by the position where a lateral root or its primodium was present or being formed. Therefore, RAV2 might, in some way, be involved in the regulation of lateral root formation. Although extensive histological studies have been reported about lateral root development (Malamy and Benfey, 1997). It is totally unknown how the position of lateral root formation is determined. Further detailed analyses of RAV2 as to its expression and function may provide useful information about the mechanism for determination of lateral root initiation. The association of RAV2 with lateral root formation is again reminiscent of *TCH4* gene, which encodes xyloglucan endoglycosyltransferase, because it is strongly expressed in lateral root primodia (Xu *et al.*, 1995).

Although the present study did not provide a clear image of the function of RAV2, the results presented here, when combined with other future studies, such as the isolation of T-DNA insertional mutants and analyses with sense and antisense transgenic plants, should lead to the elucidation of its physiological function. Since RAV1 appears to play a redundant or closely related function, parallel studies on the function and expression of RAV1 will also be important.

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