A Serine/Threonine Protein Kinase Gene Isolated by an in vivo Binding Procedure Using the Arabidopsis Floral Homeotic Gene Product, AGAMOUS

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During the course of characterizing fragments bound to an Arabidopsis floral homeotic protein AGAMOUS in vivo, a gene encoding a putative serine/threonine protein kinase was found on one of the fragments. The deduced 426 amino acid residues of the gene, named APK2a, are 65% identical to a previously reported Arabidopsis serine/threonine protein kinase, APK1a. The gene is composed of 6 exons and maps at 10 cM from the upper end of chromosome 1. Northern hybridization experiments indicated that the gene is strongly expressed in leaves, moderately in roots, and very weakly in flowers. Further in situ analysis of the expression in floral buds showed that the APK2a gene is expressed at pedicels, is not expressed at the floral organ primordia of wild type floral buds, but is moderately expressed in the floral organ primordia of the agamous mutant. In vitro binding assay suggests that the AGAMOUS protein binds to a sequence similar to, but different from, the known MADS-binding consensus sequences, the CArG box, located 3' downstream of the APK2a gene. These results suggest that APK2a gene expression is negatively regulated by the AG protein.

A close homologue of the APK2a gene, named APK2b, was also isolated from the Arabidopsis cDNA library. The expression pattern of the APK2b gene differs from that of APK2a. It is strongly expressed in leaves, moderately in flowers, and weakly in roots.

Key words: AGAMOUS — Arabidopsis — immunoenrichment — In vivo — Kinase — Target gene.

During the development of higher organisms, including plants, precise gene expression and signal transduction are required for proper morphogenesis. Genetic identification and the subsequent molecular analysis of developmental genes in Arabidopsis have shed light on the importance of transcription factors and several molecules involved in the signal transduction pathway, such as protein kinase. Recently, kinase genes have been cloned from Arabidopsis morphological mutants. TOUSLED (TSL), which was isolated by T-DNA tagging, provided evidence that protein kinase contributes to developmental decisions in plants (Roe et al. 1993). Mutations of TSL result in abnormal position and number of floral organs. ERECTA (ER) gene, whose defect causes inhibition of extension of the inflorescence axis, has been identified as a membrane-bound receptor kinase (Torii et al. 1996). These results indicate that an inter- or intra-cellular signal transduction pathway involving protein kinase is required for proper development of organs in Arabidopsis. Furthermore, a number of kinase genes have been isolated, based on biochemical purification or sequence similarity of a conserved kinase motif (Hanks et al. 1988, Hirayama and Oka 1992, Hwang et al. 1996). The expression patterns of these genes are different, in spite of their structural similarity. It is therefore necessary to clarify the functional relationships between these protein kinases.

Other than kinase genes, many important genes for flower development have been proved to be putative transcription factors. (for review, see Meyerowitz et al. 1991, Okada and Shimura 1994, Weigel 1995). Floral organ identities of Arabidopsis flowers are specified by a set of homeotic genes: APETALA1 (AP1), APETALA2 (AP2), APETALA3 (AP3), PISTILLATA (PI), and AGAMOUS (AG) (Goto and Meyerowitz 1994, Mandel et al. 1992, Jack et al. 1992, Jofuku et al. 1994, Yanofsky et al. 1990). All of these genes, except AP2, encode putative transcription factors with a DNA-binding motif, MADS domain. In vitro binding assay shows that the AG protein homodimer, AP1 homodimer, and AP3/PI heterodimer specifically bind to double-stranded DNA with sequences known as CArG box (Shiraiishi et al. 1993, Huang et al. 1993, Riechmann et al. 1996). The proteins may positively or negatively regulate the expression of "target genes" that work in the specification and growth of the floral organ primordia.

Abbreviations: AG, AGAMOUS; cM, centi-morgan; SSC, 0.15 M sodium chloride plus 15 mM sodium citrate.

The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession numbers D88206 for APK2a and D88207 for APK2b.

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the nature of these target genes is poorly understood, mostly because they are experimentally difficult to clone.

One of the screening procedures for target genes is to search for genes carrying the CArG box in their vicinity. In several trials of cloning genomic fragments with this sequence, however, no candidate genes were found in their neighbors. The second procedure is a genetic approach, screening mutants whose phenotype is partly overlapping with the homeotic mutants. It can be postulated that the mutated genes of the phenotype may work downstream of the homeotic genes in genetic regulatory networks. Although many possible mutants are being examined, no good candidates for target genes have yet been reported. The third possible procedure is to enrich DNA sequences bound by AG protein in native chromatin by immunopurification using antibody specific to AG protein. This method is free from unknown co-factors, which may be necessary for appropriate binding of a transcription factor to the target site, conformation of chromatin, or methylation of genomic DNA in vivo. This procedure for the recovery of DNA fragments bound to a transcription factor has been reported in Drosophila and mouse (Grabau et al. 1992, Tomotsune et al. 1993). We essentially followed the mouse protocol; samples were fixed by paraformaldehyde before the immunoenrichment to crosslink the proteins to each other and to the DNA, preventing dissociation of the binding site from the AG protein and allowing a thorough washing to exclude nonspecific or artificial associations between free DNA and AG protein (Tomotsune et al. 1993).

We found several candidates for AG protein target genes located near the binding site, including a putative serine/threonine protein kinase, APK2a. Expression of APK2a in flowers is restricted to the pedicel, suggesting that APK2a gene expression is negatively regulated by AG.

Materials and Methods

Chromatin isolation—Floral buds up to stage 10 of Arabidopsis thaliana (Lansberg erecta) were fixed with a fixative (2% paraformaldehyde, 10 mM phosphate buffer, and 0.1 M NaCl) overnight. After washing with M0 buffer (10 mM phosphate buffer and 0.1 M NaCl) and incubation in M2 buffer (10 mM phosphate buffer, 50 mM NaCl and 0.1 M sucrose) for several hours at 4°C, the sample was removed from the buffer, frozen with liquid nitrogen, ground in an earthenware mortar into powder, and suspended in M1 buffer (10 mM phosphate buffer, 0.1 M NaCl, 10 mM mercapto-ethanol, and 1 M hexylene glycol). The suspension was filtered through Miracloth (Calbiochem) and centrifuged at 1,000×g for 10 min at 4°C. The pellet, containing crude nuclei, was resuspended in M2 buffer (10 mM phosphate buffer, 0.1 M NaCl, 10 mM mercapto-ethanol, 1 M hexylene glycol, 10 mM MgCl2 and 0.5% Triton-X) and the suspension was centrifuged at 1,000×g for 10 min. This wash was repeated for four times; the last wash was done with M3 buffer (10 mM phosphate buffer, 0.1 M NaCl and 10 mM mercapto-ethanol). The crude nuclear preparation was suspended with the equal volume of Sonic buffer (10 mM phosphate buffer, 0.1 M NaCl, 0.5% sarkosyl and 10 mM EDTA) and sonicated on ice with duty control 50% and output 5 for 20 s (Branson Sonifier 250). Sonication was repeated 20 times avoiding to heating the suspension. After sonication, the suspension was centrifuged at 12,000×g for 10 min, and the supernatant was collected. It was then further purified by isopycnic centrifugation at density 1.42 for 72 hours at 40,000 rpm (Beckman SW 55T), fractionated into 10 fractions, and dialyzed against M buffer. The DNA and protein content of each fraction were examined with agarose gel for DNA and with SDS-PAGE for protein. Fraction numbers 6, 7 and 8, that contained the DNA-protein complex, were further purified by affinity column.

Affinity-purification by the anti-AG antibody column—The carboxyl-terminal part of the AG protein (from 220 to 285 amino acid residues) was expressed in E. coli cells using the glutathione S-transferase fusion protein expression system (2.5 mMTris-HCl on a glutathion column, and injected into rabbits. Anti-AG polyclonal serum was obtained after six boosts and purified on an antigen-conjugated Sepharose 4B column (Pharmacia). The fractions of the DNA-protein complex were passed through an affinity column containing anti-AG antibody-conjugated Sepharose 4B. The column was washed with three column volumes of M0 buffer, L buffer (0.1 mM phosphate buffer), M buffer (10 mM phosphate buffer and 1 M NaCl), and M0 buffer for three times each. AG protein-DNA complex was eluted with two column volumes of E buffer (10 mM phosphate buffer and 3 M NaSCN), dialyzed against L buffer, and incubated with 0.5 mg/ml protein K, 50 mM Tris-HCl (pH 8.0), and 0.1 M EDTA for 3 hours at 55°C. DNA was extracted with phenol/chloroform mixture and then with chloroform, precipitated with ethanol, and resuspended in 100 μl TE buffer.

Selection of concentrated clones—The DNA fragments were blunt-ended with T4 polymerase (Blunting Kit, Takara) and separated into two parts. One part of the DNA was ligated with EcoRI linker, cloned into the Agt 10 vector (Stratagene), and packaged using GigaPack II packaging extract (Stratagene). The other part of the DNA was ligated with lane-linker (Ko et al. 1998), amplified by PCR for 35 cycles, and labeled using the random primer and Knell Fragments (Takara). The phage clones were spotted on lawn plates in order to make the size of each plaque equal. Replica filters of the spotted library were hybridized using the amplified DNA as a probe. Phage DNA whose plaques showed strong signals were isolated.

Gel mobility shift assay—The gel mobility shift assay was performed following the procedure of Shiraiishi et al. (1993). An insert of clone 92 was digested with XhoI, XbaI, and HindIII into two fragments, F1 and F2, which were end-labeled with [α-32P] deoxy-CTP and Klenow Fragments. Three kinds of double-stranded oligonucleotides, wild-type (5'-CAGATAAATAACCTTTTAACCATATTTT-3') and mutant 2 (5'-CAGATAAATAACCTTTTAACCATATTTT-3'), were chemically synthesized and end-labeled with [γ-32P] ATP at both ends. Translated AG protein was overproduced in E. coli BL21 (DE3) harboring pET-AG310- myc (Shiraiishi et al. 1993). Radiolabeled probes of 1×10^6 cpm were incubated with 4 μg E. coli extract with or without truncated AG protein in Binding buffer (10 mM HEPES-NaOH (pH 7.9), 1 mM EDTA, 1 mM DTT, 5% glycerol, 30 mM KCl, 1 μg μl−1 Poly(dl-SC), and 5 μg μl−1 BSA) for 40 min at 25°C. The super-shift was done using the monoclonal antibody 9E10 (Shiraiishi et al. 1993). The DNA-protein complex was separated using 4% PAGE (40 : 1 acrylamide : bio-acrylamide). The results were visualized and quantitated with the BAS-1500 Imaging System (Fuji Photo
Mapping of clone 92—The chromosomal locus of clone 92 was determined using recombinant inbred (RI) lines (Lister and Dean 1993). Genomic Southern blots using ecotypes Columbia and Lansberg erecta DNA were done after digestion of genomic DNA with EcoRI. Hybridization was performed with the 5 kb HindIII genomic fragment corresponding to clone 92. Genomic Southern blots of 48 RI lines were hybridized with the same probe, and the map position was determined using software Mapmaker version 2.0 (E.L. Dupont de Nemours).

DNA and RNA gel blot—DNA gel blot analysis was performed using 1.5 μg of whole-plant DNA, digested with EcoRI, HindIII and BamHI, electrophoresed on 1% agarose gel, and blotted onto Hybond-N nylon membranes according to procedures recommended by the manufacturer (Amersham). The DNA blots were washed with 0.1 x SSC at 50°C as moderate stringency. For the RNA gel blot, 0.5 μg of poly(A) RNA was denatured and run on formaldehyde agarose (1%) gels, followed by transfer to Hybond-N nylon membranes. As a size marker, an RNA ladder (GIBCO BRL) was run on the gel. Hybridization was carried out in a solution 50% formamide and 6×SSC at 42°C overnight. The blots were finally washed with 0.2 x SSC at 65°C as high stringency.

Screening and sequencing of genomic and cDNA clones—The Arabidopsis thaliana (Lansberg erecta) genomic library was screened to isolate the flanking sequence of clone 92. About 80,000 plaques were screened by [32P]-labeled 180 bp EcoRI fragment of clone 92 as a probe, and five clones were isolated. A 700 bp HindIII genomic fragment corresponding to clone 92 was subcloned into pBluescript SK+ (Stratagene). Arabidopsis thaliana (Lansberg erecta) flower and leaf cDNA libraries constructed with AZAPIII were screened with the 700 bp HindIII fragment as a probe. Positive clones were restriction-mapped, and the clones containing large inserts were subcloned into pBluescript II SK+ for sequencing. Sequencing was performed using a Taq Dye Primer Cycle sequencing kit (Applied Biosystem) on an automated DNA sequencer (model 373A; Applied Biosystems). Sequences were analyzed through E-mail using the Genetyx-Mac sequence analysis program (SDC), and homology searches were analyzed using MFSrch (University of Edinburgh), based on the Smith and Waterman full dynamic algorithm.

In situ hybridization—In situ hybridization was carried out by following essentially the procedure of Wakamatsu and Kondoh (1990). Floral buds were fixed overnight with a fixative (4% paraformaldehyde, 10 mM phosphate buffer and 0.1 M NaCl), dehydrated gradually, and finally immersed in 100% ter-butanol. They were embedded in paraffin and sectioned with a microtome at a width of 6 μm. The sections on slide glasses were treated with 0.2 M HCl and 1 μg ml−1 proteinase K and then acetylated. RNA probes were synthesized in vitro by transcription of linearized 92-3 cDNA clone with 500 MBq [35S] UTP by T7 polymerase or T3 polymerase (Toyobo). Hybridization was done for 20 hours at 50°C in a mixture containing 50% formamide, 2× SSC, 10 mM EDTA, 10 mM DTT, 6× Denhard’s solution, 10% dextran sulfate, 0.5 mg ml−1 denatured salmon sperm DNA, 0.5 mg ml−1 yeast tRNA, and 2×10⁶ cpm μl−1 [35S]-labeled probes. After hybridization, the samples were treated with 20 μg ml−1 RNase A for 30 min at 37°C, washed with the 10 mM DTT, 0.1× SSC, 20% formamide at 50°C overnight, exposed with NTB-2 emulsion (Kodak) for two weeks at 4°C, and developed with Konica dol X (Konica).

Results

Isolation of genomic fragments bound by AG protein in vivo—Soluble nucleoprotein complexes were prepared from paraformaldehyde-fixed floral buds and passed through the anti-AG antibody-conjugated column. After immunoenrichment, a library of purified DNA was constructed using the λgt10 vector. The library was plated and hybridized using the same immunoenriched DNA as a probe. Nearly 200 plaques were screened, and five plaques with strong signals were taken. In order to examine whether the isolated clones represented immunoenriched sequences or repetitive sequences in the Arabidopsis genome, we did a Southern blot analysis for each clone. As shown in Figure 1, clones 34, 88, and 98 seemed to include highly repetitive sequences, because they showed strong and multiple bands. Clone 144 might have had middle repetitive sequences because several bands were hybridized. The situation was different, however, with clone 92. Although clone 92 showed a strong signal when hybridized with the immunoenriched DNA, it could not carry repetitive sequences, because it showed two bands for each enzyme (Fig. 1c). It is strongly suggested, therefore, that this clone is enriched in the library.

In order to demonstrate that an insert fragment of clone 92 was specifically enriched in the library, a replica filter, carrying independent 1,000 plaques of the library,
was hybridized with clone 92. As the average length of inserts were 500 bp, the inserts from the 1,000 immunoenriched clones represented 0.5% of the *Arabidopsis* genome. The probability that a unique genome sequence will appear more than twice in 1,000 random clones is less than $3 \times 10^{-5}$. Clone 92 was concentrated to the same extent in two independent immunoenriched libraries, but not in an analogous library selected by control antibody affinity column. The existence of unique sequences hybridizing with multiple plaques, therefore, provides strong evidence for enrichment of AG protein binding sites in the phage library and indicates that it presumably represents a genomic fragment bound by AG protein in vivo.

**in vitro binding of AG protein to clone 92**—We then examined whether the truncated AG protein (MADS domain and I domain, which are sufficient regions for dimer formation and specific binding to DNA) (Shiraishi et al. 1993, Riechmann et al. 1996) binds to clone 92 in vitro. The insert of clone 92 (300 bp) was digested with *HincII* to two fragments, F1 (140 bp) and F2 (160 bp) (shown in Fig. 4), labeled, and incubated with or without the AG protein. The binding complex was separated from naked DNA on polyacrylamide gels. As shown in Figure 2a, the F1 fragment was shifted upward when incubated with an *E. coli* lysate containing the AG protein, but not when incubated with a control lysate (lanes 1–3). This result indicates that the AG protein binds to the F1 fragment in vitro. The binding was certified by a super-shift band observed when the antibody to the AG protein was added to the reaction mixture (Fig. 2b, lane 3). There is no evidence for the binding

![Fig. 2 In vitro binding of AG protein to clone 92. The insert of clone 92 was cut out with *XhoI*, *XbaI*, and *HincII* to two fragments, F1 and F2. F1 and F2 were $^{32}$P-labeled and incubated with or without truncated AG proteins followed by gel mobility shift assay. AG-DNA complex was separated with 4% polyacrylamide gel electrophoresis. (a) F1 probe was electrophoresed without *E. coli* extract (lane 1). F1 probe was electrophoresed after incubation with the extracts of *E. coli* BL21(DE3) harboring pET3a (extract without truncated AG protein) (lane 2) or *E. coli* BL21(DE3) harboring pET-AG310-myc (extract containing the truncated AG protein) (lane 3). F2 probe was electrophoresed without *E. coli* extract (lane 4). F2 probe was electrophoresed after incubation with the extract without the AG protein (lane 5) or with the extract containing the AG protein (lane 6). (b) F1 probe was electrophoresed without *E. coli* extract (lane 1). F1 probe was electrophoresed after incubation with the extracts containing the AG protein (lane 2) or with the extracts containing the AG protein and the monoclonal antibody, 9E10 (lane 3). 9E10 specifically recognize the truncated AG protein produced by *E. coli* BL21(DE3) harboring pET-AG310-myc.

![Fig. 3 In vitro binding of AG protein to wild type- and two mutated-synthesized nucleotide probes. Gel mobility shift assay was performed with wild type probe (W) found in clone 92 and two mutated probes (M1 and M2). W probe was electrophoresed without *E. coli* extract (lane 1). W probe was electrophoresed after incubation with extracts containing the AG protein (lane 2) or with extracts containing AG protein and the monoclonal antibody, 9E10 (lane 3). Incubation of W probe and the extracts containing the AG protein was performed with various amounts of W, M1, or M2 unlabelled competitor: 20-fold unlabelled W (lane 4), 200-fold unlabelled W (lane 5), 20-fold unlabelled M1 (lane 6), 200-fold unlabelled M1 (lane 7), 20-fold unlabelled M2 (lane 8), 200-fold unlabelled M2 (lane 9). M1 probe was electrophoresed without *E. coli* extract (lane 8) or electrophoresed after incubation with the extracts containing the AG protein (lane 9). M1 probe was electrophoresed without *E. coli* extract (lane 10). M1 probe was electrophoresed after incubation with the extracts containing the AG protein (lane 11) or with extracts containing AG protein and the monoclonal antibody, 9E10 (lane 12). M2 probe was electrophoresed without *E. coli* extract (lane 13) or electrophoresed after incubation with extracts containing the AG protein (lane 14). Triangle shows binding of unknown *E. coli* protein to clone 92. It was observed with *E. coli* extract without the AG protein (data not shown).
of the AG protein to the F2 fragment (Fig. 2a, lane 4–6). It is concluded, therefore, that the F1 fragment includes AG protein-binding sequences. Sequence analysis of the fragment suggests that the most probable DNA sequences is 5'-TAACCTTTTAACCCAT-3'. This sequence is similar to the previously determined AG-binding consensus sequence; 5'-TT(A/T/G)CC(A/T)6GG(A/T/C)AA-3', known as CArG box (Shiraishi et al. 1993, Huang et al. 1993). However, there is a critical difference between the CArG box and the sequence found in the F1 fragment. The CArG box has a palindromic structure; six As or Ts are inserted between two Cs and two Gs, but the two Gs are replaced by two Cs in the F1 fragment. It was important to examine whether the AG protein could bind the CArG-like sequences in vitro, because the palindromic structure of the CArG box has been considered to be necessary for the binding of AG protein as a dimer.

We used synthesized double DNA strands containing the original DNA sequences found in the F1 fragment (wild type) or two types of altered sequences to perform the gelshift assay of the AG protein. The altered sequences contain CC(A/T)GG (M1) or GG(A/T)CC (M2) (Fig. 3). These changes in the M1 sequence make it match the CArG box consensus sequence. As shown in Figure 3, the M1 fragment shows both shift and supershift bands, indicating that the AG protein binds to the CArG box (lanes 10–12). When the original sequence of the F1 fragment (wild type) was used, a clear shift band and a supershift band were observed, although the band strength is approximately 40 times weaker than that of M1 fragment (lanes 1–3). The shift band was diminished when excess amounts of unlabelled wild type fragment or M1 fragment were added (lanes 4–7). With the M2 fragment, no binding was observed (lane 13 and 14). These results strongly indicate that AG protein binds specifically to the wild type fragment found in clone 92, although it does not completely fit the CArG box.

The shifted band size of the wild type fragment was the same as that of M1 fragment (lane 2 and 11). AG protein binds to the CArG box as a dimer in vitro (Huang et al. 1995). These results indicate that AG protein also binds to clone 92 as a dimer.

Search for the neighboring genes—In order to examine whether clone 92 contained parts of genes or of regula-
Kinase genes in Arabidopsis

RNA dot blot analysis was done using the 700 bp HindIII fragment corresponding to clone 92 (shown in Fig. 4) as a probe. Some positive signals were found in total RNA samples prepared from leaves and from flowers (data not shown). We screened cDNA libraries constructed from flower and leaf tissues and isolated 6 clones. To our surprise, the isolated cDNA clones are separated into two genes designated at92-2 and at92-3. Homology analysis of the predicted amino acid sequences of the at92-3 gene revealed that it contains a region with strong homology to the Arabidopsis protein kinase APKla (described below). Therefore, we named the at92-3 gene “APK2a”. The at92-2

(a) APK2a
(b) APK2b
(c) APKla
(d) APKlb

Fig. 5 Amino acid sequence alignment of protein kinases. (a) The amino acid sequence alignment of APK2a, APK2b, APKla and APKlb. Identical amino acid residues with APK2a sequences are indicated by +. Hyphens indicate unpublished amino acid residues (APKlb) and gaps introduced to maximize alignment. Subdomain I to XI of protein kinase are shown with horizontal bars. (b) The amino acid sequence alignment of APK subfamily, APKla, APKlb (Hirayama and Oka 1992), NAK (Moran and Walker 1993), ARSK1 (Hwang and Goodman 1995), AK14, AK13 (Thummler et al. 1995) in the catalytic domains, VI-B, VII, VIII, and IX. Identical amino acid residues with APK2a sequences are indicated by *. Hyphens indicate gaps introduced to maximize alignment. Numbers show the percentage sharing identical amino acids in each amino acid sequences.
Kinase genes in Arabidopsis

gene encoding 655 amino acid residues has no homology to known genes, but it includes four acidic regions made of glutamate and aspartate repeats (manuscript in preparation). Genome structure of the APK2a and at92-2 genes was clarified by obtaining genomic clones covering about a 6 kb region. As shown in Figure 4, the at92-2 gene and APK2a gene are located in opposite directions and overlap at their 3' ends. Clone 92 is located at 3' region of each gene: 160 bp downstream of the poly(A) additional site of APK2a gene and in the 5th intron of at92-2 gene. This region mapped on the short arm of chromosome I between g3786 and g3829 (Fig. 4).

Structure and expression pattern of APK2a gene

The APK2a cDNA has an open reading frame of 1,278 bp, encoding a protein of 426 amino acids with a calculated molecular weight of 46.3 kDa (Fig. 5a). The putative kinase domain of this protein is flanked by short non-kinase domains on both sides. As APK2a contains no membrane spanning region or extra membrane domain, it is likely that it is either a cytoplasmic or nuclear kinase. As shown in Figure 5, the kinase domain of APK2a has all 11 conserved subdomains of eukaryotic protein kinase and all the invariant amino acid residues in their proper positions (Hanks et al. 1988).

The expression pattern of APK2a was investigated by Northern blot analysis. As shown in Figure 6, the APK2a gene is strongly expressed in leaves, moderately in leaves, but barely in flowers. In situ hybridization analysis has shown that APK2a gene is expressed at very low levels in floral organ primordia, but at relatively high levels in pedicels in young buds of stages 4–8 (Fig. 7a). Expression of APK2a gene is localized in the peripheral zone of the pedicel at stage 7, and it stops in later stages (stage 9–). In agamous (ag-1) mutant flowers, the APK2a signal was detected uniformly in floral organ primordia and in pedicels at stages 3–8 (Fig. 7b).

Isolation of APK2b gene

During the screening of APK2a cDNA clones, several clones with weak signals were obtained. These clones were found to encode a new protein kinase of 426 amino acid residues, named APK2b. APK2a and APK2b is highly homologous; 75% homology in the nucleotide sequences at the coding region, 83% homology in the deduced amino acid sequences, and 100% matches in the 11 kinase subdomains (Fig. 5a). APK2b also contains no membrane spanning region or extra membrane domain. The expression pattern of the APK2b gene is different from that of APK2a gene (Fig. 6b). APK2b gene is strongly expressed in leaves, moderately in flowers, but only weakly in roots. In the protein sequences database, over 60 Arabidopsis kinase genes are registered. APK2 gene products are closest to APK1 gene products, a serine/threonine protein kinase with unknown function that was cloned using PCR (Hirayama and Oka 1992), NAK1 (Moran and Walker 1993), ARSK1 (Hwang and Goodman 1995) and AK 13, 14 (partial sequence for highly conserved subdomains VI-B, VII, VIII, and IX by PCR) (Thummler et al. 1995). Figure 5b shows the amino acid sequence alignment of highly homologous kinase genes in the catalytic domains, VI-B, VII, VIII, and IX. These domains are responsible for substrate specificity (Hanks et al. 1988). Close inspection of the amino acid sequences of subdomains VI and VIII indicates that APK2a and APK2b have serine/threonine substrate specificity (Fig. 5b). These eight highly homologous kinase genes may represent one subfamily among the over 60 kinase genes.

Discussion

This paper describes a new protein kinase gene cloned from an “immunoenriched” library; a genomic library of DNA fragments that are bound to AG protein in the nuclei of cells of floral organ primordia. This procedure was previously used to isolate target genes of transcription factor in Drosophila and mouse embryo (Graba et al. 1992, Tomotsune et al. 1993). Our procedure was essentially the same as the procedure used by Tomotsune et al. (1993), but we added a step which purified the nuclei in order to remove chloroplast genome DNA that could hinder the screening step.
Kinase genes in *Arabidopsis*

Fig. 7  In situ hybridization of APK2a in wild type and *ag-1* inflorescence sections. Longitudinal sections of wild type (a) and *ag-1* (b) were hybridized with [35S]-labeled antisense probe. (c) Longitudinal section of wild type hybridized with sense probe. Photographs were taken using bright field (upper) and dark field (lower). The number shows the stage of flower development (Smyth et al. 1990). pe: pedicel.

by contamination. We have examined nearly 200 clones of the "enriched" library, whether they are enriched in the library or not. We have obtained 5 concentrated clones, but four of them were shown to contain multicity DNA sequences, showing that repetitive clones represent about 2% of this enriched library. In the Arabidopsis nuclei genome, repetitive sequences represent about 10% (Martinez-Zapater and Somerville 1986). The lower proportion of repetitive sequences supports the enrichment of this library. We selected one clone, clone 92, because it was significantly enriched in the library.

Genetic analysis has shown that *AG* has plural functions in flower development (Bowman et al. 1991, Sieburth et al. 1995, Mizukami et al. 1995). *AG* prevents *API* RNA accumulation in the central part of wild-type floral organ primordia (Gustafson-Brown et al. 1994). We screened the immunoenriched library with *API* genomic fragments as a probe in order to investigate whether the genomic sequences in the vicinity of *API* gene are enriched in the library. There was no obvious enrichment of *API* genomic sequences (data not shown). This result suggest that *API* is not a direct target of *AG* protein. As there is a strong presumption that *AG* has plural target genes, it is quite possible that there are some more enriched clones in addition to clone 92 in the enriched library. Further screening is now underway. Furthermore, we must determine whether the sequences containing the AG-binding consensus sequence, CArG box, are enriched in the library.

APK2a gene has a stretch of nucleotide sequences in the 3’ downstream region, where *AG* protein can bind in vitro. These binding sequences, 5’-TAACCTTTAACC CAT-3’, do not match the CArG box. When we replaced CC(A/T)CC with CC(A/T)GG to match the CArG box, the binding affinity was strengthened about 40 times. This result indicates that the binding affinity of *AG* protein to the 3’ downstream region of APK2a gene is relatively low in vitro. Questions that may be raised are whether the APK2a gene is a true target gene of *AG* protein and whether the low binding affinity is sufficient for correct recognition by *AG* protein.

The expression pattern of the APK2a gene is antagonistic to the expression pattern of the *AG* gene. As already reported, *AG* is expressed in the central part of floral organ primordia and not in other tissues (Drews et al. 1991, Bowman et al. 1991). As shown in Figures 6 and 7, the APK2a gene is expressed in all organs including pedicles, but not at the center of floral organ primordia. In addition, the expression of APK2a gene is observed uniformly throughout the entire floral organ primordium of *ag-1* mutants. These results suggest that APK2a gene ex-
pression is negatively regulated by AG protein.

There are two possible models explaining how AG protein controls APK2a gene expression in spite of its weak binding affinity to the binding sequences. The first one is that other factors facilitate correct and strong binding in vivo. In the cases of SRF and MCM1, both transcription factors carrying MADS domains, homeodomain proteins are known to enhance the DNA-binding activity of the MADS proteins (Bender and Sprague 1987, Jarvis et al. 1989, Grueneberg et al. 1992). MCM1 functions in cooperation with accessory factors that impart specific responses for cell types, α and α, to different upstream activating sequences (UAS). The MCM1 protein cooperates with the MATα1 protein, a homeodomain protein which activates α-specific genes and with the MATα2 protein, also a homeodomain protein which represses α-specific genes, thereby establishing the cell type-specific patterns of gene expression in the two haploid cell types of yeast. Although the complexes of MCM1 and MATα1 bind to UAS of STE3 gene, MCM1 does not bind to the UAS in the absence of MATα1 (Bender and Sprague 1987). Phox-1, a homeodomain protein enhances the binding of SRF to a serum response element of c-fos (Grueneberg et al. 1992). AG protein could function in cooperation with accessory factor(s) which enhance the binding activity in vivo. It is of interest to examine proteins interacting with AG protein in nuclei. The second possibility is that there may be several transcriptional regulatory systems dependent on AG protein concentrations in nuclei. Analysis of transgenic plants containing AG antisense DNA has shown that two functions of AG protein, floral meristem determinacy and reproductive organ identity, require different levels of AG activity (Mizukami and Ma 1995). Higher levels of AG activity are required for floral meristem determinacy than for the organ identity. These data might reflect the binding activity between regulatory elements and AG protein. If the binding activity of AG in vitro reflects that in vivo, the regulation of APK2a by AG protein needs more AG activity than that of a gene regulated by the CArG box. The ratio of the binding in vitro, however, would not always reflect that of in vivo, because the binding in vivo is not free from the conformation of chromatin.

The putative AG protein binding site is located 160 bp downstream of the poly(A) additional site of APK2a gene. This suggests that the signal of AG binding may be transferred a long distance to the promoter region. Similar regulatory systems are known in both animals and plants. A negative regulatory cis-element is located in the 2nd intron of PLENA gene of Antirrhinum majus (Bradley et al. 1993). Tissue-specific positive regulatory elements are located in the introns of chicken δ-crystallin gene (Hayashi et al. 1987), Arabidopsis EF-1a (Curie et al. 1993), and Oat phyA3 gene (Bruce and Quail 1990). Mouse Wnt-1 gene has a cis-acting 3' enhancer element that confers correct temporal and spatial expression (Echelard et al. 1994). In the APK2a gene, the putative AGAMOUS binding region might function as a negative regulatory cis-element. A close investigation of the regulatory mechanism is underway using transgenic plants.

APK2a protein may have kinase activity, because it is highly homologous to the known serine/threonine protein kinase. If the APK2a gene is negatively regulated by AG protein, it may be necessary to stop expression of the protein kinase to permit the floral organ development directed by AG protein. Although the mechanism is totally unknown, there are several cases in which protein kinases are involved in organ development in both plants and animals. An Arabidopsis gene, TOUSLED (TSL), encodes a protein kinase. A mutation in TSL results in abnormal position and number of floral organs (Roe et al. 1993). Another Arabidopsis gene, ERECTA (ER), whose mutation causes reduced cell elongation in leaves, inflorescence axes, and siliques, encodes a membrane-bound receptor kinase (Torii et al. 1996). The Drosophila segment polarity gene, shaggy, required for developmental processes from embryo to larvae and adults, encodes a serine/threonine protein kinase (Bourouis et al. 1990, Heitzler and Simpson 1991). Lack of shaggy gene product leads to homeotic transformation of hair cells into sensory bristles, showing that shaggy functions as a homeotic gene (Heitzler and Simpson 1991). It seems reasonable, therefore, that APK2a has a role in signal transduction pathways on flower development as a target of AG protein. It is also necessary to examine the detailed expression patterns of APK2a spatially and temporally in wild type and various ag mutant flowers.

APK2a and APK2b share striking sequence similarities with each other (Fig. 5) and are assigned to one novel subfamily of serine/threonine protein kinase genes in Arabidopsis. The expression patterns of APK2a and APK2b differ each other. They may function in different cells, controlling branches of the vegetative and floral morphogenesis regulatory hierarchy. Although AtATase2a and AtATase2b are probably both protein kinases, their different expression patterns suggest that they control different signaling cascades. It is necessary to examine target proteins in order to understand their roles in the developmental processes of Arabidopsis.

It is important for understanding the molecular mechanisms of organ development to identify and clone target genes of transcription factors encoded by regulatory genes, such as the floral homeotic gene, AG. Genetic analyses indicate that several groups of target genes exist. This report is the first biochemical isolation of a possible target gene. Following experiments will reveal the whole scope of target genes and the regulatory mechanisms of developmental processes of flowers.
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References


Kinase genes in *Arabidopsis*


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