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Functional cloning of a cDNA encoding Mei2-like protein from *Arabidopsis thaliana* using a fission yeast pheromone receptor deficient mutant

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Abstract To isolate *Arabidopsis* cDNAs that encode signal transducers and components involved in the regulation of meiosis, a trans-complementation analysis was performed using a *Schizosaccharomyces pombe* meiosis-defective mutant in which the genes for pheromone receptors were disabled. One cDNA obtained in this screening encodes a polypeptide, named AML1, that shows significant similarity to *S. pombe* Mei2 protein and has three putative RNA-recognition motifs like as Mei2. Mei2 is involved in the regulation of meiosis in fission yeast. Northern blot analysis showed that the *AML1* gene is expressed in each organ. The possible functions of AML1 are discussed.

Key words: *Arabidopsis thaliana*; *Schizosaccharomyces pombe*; Trans-complementation; Meiosis induction; RNA-recognition motif

1. Introduction

Trans-complementation using a yeast mutant as a host is a useful strategy not only to isolate genes of higher eukaryotes that are functionally related to the mutated genes of yeast but also to characterize the gene products whose activity can not be assessed easily in vitro. Two yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, are well characterized, especially in cell-cycle-control, control of meiosis, intracellular signal transducing mechanisms, control mechanisms for cell polarity, vesicle transport control, etc. Using mutants of these two yeasts as hosts, many genes involved in various cellular functions have been cloned from several organisms, including higher plants.

The entry into meiosis from the mitotic cell cycle is the sole means of cell differentiation for single cell organisms, such as yeast. The mechanisms for the initiation of meiosis in yeast have been studied extensively with genetic approaches, and many mutants exhibiting meiosis or sporulation deficiency have been isolated and characterized. These studies have revealed that the initiation of meiosis of *S. pombe* cells is tightly regulated by an elaborate system (for review [1]). Among predicted components in the initiation of meiosis, Mei2 has been thought to be a key protein for switching the mitotic cell cycle to meiosis. Mei2 has three putative RNA-recognition motifs (RRM) and actual RNA binding activity that is necessary for Mei2 function [2,3]. The activity of Mei2 is thought to be regulated at the transcriptional and post-translational level. The transcription of the *mei2* gene is induced when diploid cells are starved of nutrients. However, phosphorylation of Mei2 by Pat1 protein kinase represses its activity [4]. *S. pombe* cells produce two types of sex pheromone, M and P factors, which initiate the process of mating and meiosis. The signals of P or M factor are perceived by a pheromone receptor located at the plasma membrane and transduced to the nucleus through signal transducers, including a hetero-trimeric G protein (Gpa1) and protein kinases in a MAP kinase cascade (Byr2-Byr1-Spk1). The pheromone signal induces the expression of the *mei3* gene whose product binds to Pat1 directly and inhibits its kinase activity. Inhibition of Pat1 results in the activation of Mei2 and cells entry into meiosis.

Meiosis is a key event of sexual reproduction for other eukaryotes including plants. The mechanisms of the development of reproductive organs in plants have attracted much attention (for review [5–7]). Genetical analysis has been applied to study meiosis and several mutants that affect gametophyte development have been isolated and characterized [8–12]. Another approach has also been undertaken to assess the mechanisms involved in gametophyte development by identifying genes that are expressed in the specific stage of meiosis and of gametogenesis [13,14]. However, the mechanisms for gametophyte development, especially for the initiation of meiosis, remain largely to be uncovered, due to difficulties in isolating mutants that affect the initiation of meiosis.

We made an attempt to isolate *Arabidopsis* cDNAs that rescue the meiosis defective phenotype of a *S. pombe* pheromone receptor deficient mutant (RL190; *map3::ura4*$^+$/*map3::ura4*$^+$, *mam2-A48/mam2-A48*) [15,16]. In this screening, we expected that we would isolate cDNAs encoding components involved in the commitment to meiosis in plants as well as cDNAs encoding signal transducers which can bypass the pheromone signaling pathway when over-expressed in yeast cells. We cloned several cDNAs with this strategy. We describe here one cDNA with high ability to suppress the phenotype of the pheromone receptor deficient mutant. The

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deduced amino acid sequence of the putative protein encoded by this cDNA shows significant similarity to that of *S. pombe* Mei2 protein. To our knowledge, this is the first report on the *mei2*-like gene in a multicellular organism. This study shows that the trans-complementation strategy using yeast as a host can be applied to the study of the regulatory mechanisms of meiosis in plants.

2. Materials and methods

2.1. Yeast and Arabidopsis lines

A diploid *S. pombe* strain RL190 (h^{90}/h^{90}, *leu1/leu1*, *ade6-M216/ade6-M210*, *map3::ura4*$^+$*/map3::ura4*$^+$*4*, *mam2-A84/mam2-A84*) was used for trans-complementation screening. JZ127 (h^{90}, *ade6-M216*, *ura4-D18*, *leu1*, *mei2::ura4*$^+$) and JY371 (h^{90}, *ade6-M216*, *leu1*, *mei2-33*) were used in complementation assay. *Arabidopsis thaliana* (Columbia ecotype) was used as the source for construction of a cDNA library.

2.2. Constructing cDNA library

RNA was extracted from plant material including leaves, flowers and shoots using Extract-A-Plant RNA Isolation Kit (Stratagene) according to manufacturer's instructions. mRNA Purification Kit (Pharmacia Biotech) and λ ZAP cDNA Synthesis Kit (Stratagene) were used to isolate poly(A) RNA and to synthesize cDNA, respectively. The synthesized cDNA was introduced in the *Sma*I-*Sal*I site of the expression vector pREPH1, in which the *Pst*I site of the original pREP1 [17] had been deleted and the multiple cloning site (*Nde*I-*Sal*I-*Bam*HI-*Sma*I) had been converted to *Sma*I-*Bam*HI-*Sal*I-*Pst*I. The cDNA library was amplified once in *E. coli* cells.

2.3. Transformation of S. pombe cells and screening conditions

A cDNA library was introduced into the cells of strain RL190 by the LiAc method described elsewhere [18]. Transformed cells were incubated on SSA [19] thiamin minus medium at 28°C for 4 days. Colonies were exposed to iodine vapor and dark-brown stained colonies were selected, since spores are stained dark-brown by iodine. Total DNA was isolated individually from selected transformants and introduced into *E. coli* cells. The recovered cDNA clones were then subjected for further screenings. Positive cDNAs were excised from the vector DNA and ligated to pAS248 having a constitutive *adh* promoter, and the resultant plasmids were reintroduced to RL190 cells. Transformed cells were cultured on SSA-agar at 26°C for 4 days. The cells were suspended in water and fixed in 3.4% formaldehyde and subjected to microscopic observation. pDB52 that harbored the *mam2*$^+$ gene encoding P-factor receptor was used as a positive control [15]. The efficiency of induction of meiosis was determined by counting spore-forming cells. Positive cDNAs were inserted into pSKII vector for nucleotide sequencing.

2.4. Southern and Northern blot analysis

Genomic Southern blot and Northern blot analyses were performed as described previously [20].

3. Results

3.1. Isolation of cDNA clones that have the ability to rescue the sporulation defective phenotype of RL190

We chose a trans-complementation assay using a fission yeast mutant to obtain plant cDNAs that encode functional components implicated in the signal transduction or the initiation of meiosis. In *Schizosaccharomyces pombe*, the initiation of meiosis requires several conditions including; diploidy of the cell, nutrients starvation, and activation of the pheromone signaling system [1]. *S. pombe* has two types of pheromone receptors, the receptor for M-factor and the receptor for P-factor, encoded by two genes, *map3* and *mam2*, respectively. In RL190 cells, these two genes for pheromone receptors were disrupted or disabled. Thus, the cells of RL190 cannot enter meiosis even under starvation conditions. A cDNA library derived from adult plants of *Arabidopsis thaliana* constructed in a *S. pombe* expression vector was introduced in RL190 cells to isolate cDNA clones that conferred the ability to induce sporulation. Nine potential cDNA clones have been isolated in this screening. The efficiency to induce sporulation in *S. pombe* varied among these cDNAs (data not shown). The clone AR301 had the highest efficiency of spore formation and was further analyzed.

3.2. AR301 encodes a Mei2-like protein

Determination of the nucleotide sequence of AR301 cDNA revealed that this cDNA was truncated. Using AR301 cDNA as a probe, we isolated several cDNAs from a cDNA library constructed in a lambda phage vector. A cDNA, about 3.6 kb length, was sequenced and shown to contain the nucleotide sequence identical to AR301 cDNA (Fig. 1). This cDNA has a putative open reading frame encoding a polypeptide that consists of 884 amino acid residues. The 5′ non-coding sequence is rather long (ca. 450 bp). Since there are several in-frame stop codons upstream of this open reading frame, this cDNA appears to encode a full-length polypeptide. The deduced amino acid sequence of the predicted protein shows significant similarity to *S. pombe* Mei2 protein (Fig. 2). The highest similarity, 65%, is located in the C-terminal region, additional similarities are found around putative RNA-recognition motifs (RRM). *S. pombe* Mei2 protein has three RRMs and possesses actual RNA binding activity [3]. We found three putative RRM in the full-length AR301 protein at the nearly same positions as found in *S. pombe* Mei2 (Fig. 2). Because of the high similarity in structure, we named this *Arabidopsis* protein AML1 (*Arabidopsis* Mei2-like protein). Recently, the phosphorylation sites of Mei2 by Pat1 kinase have been determined, which were Ser^{438} and Thr^{527} [4]. Corresponding amino acid residues cannot be found in AML1, suggesting that at least the upstream regulation device for AML1 is different from that of *S. pombe* Mei2.

3.3. AML1 could suppress the meiosis-deficient phenotype of RL190, but not of the mei2 disruption mutant

We determined the spore formation efficiency of the AR301 cDNA and the AML1 cDNA that contained truncated or full coding region, respectively. These two cDNAs were inserted downstream of a constitutive *adh* promoter in pAS248 to construct pAH6 (AR301 cDNA) and pAH27 (full-length AML1 cDNA). These plasmid DNAs were introduced into RL190 cells. Transformed cells were grown on SSA agar plate for 4 days at 26°C. Table 1 summarizes the sporulation efficiency. The AR301 cDNA and the full-length AML1 cDNA induced sporulation but their efficiencies were lower than that of the *mam2*$^+$ gene. It is noteworthy that there was no

Table 1
Spore formation efficiency of AR301 and AML1 cDNA

Plasmid	Efficiency (%)[a]
pDB52 (*mam2*$^+$)	12.43 ± 4.29 (n = 6)
pAH6 (AR301)	2.63 ± 1.33 (n = 8)
pAH27 (AML1)	1.85 ± 0.64 (n = 6)
pAS248	0.17 ± 0.10 (n = 6)

[a]The average ± S.D. for the indicated number of preparations (n) is given. Each preparation contained 200 ~ 1000 cells.

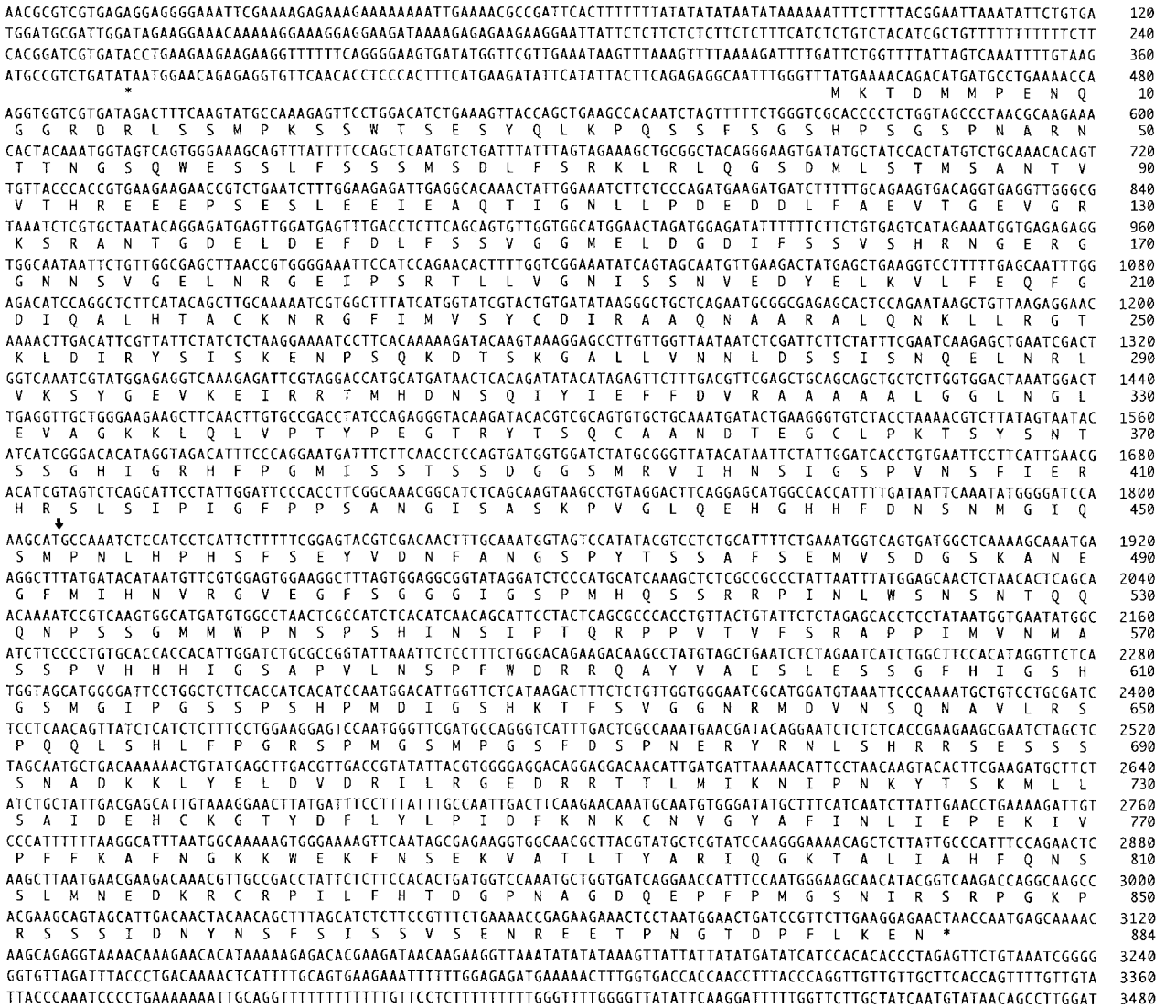

Fig. 1. Nucleotide and deduced amino acid sequences of the AML1 cDNA. The amino acid sequence of the putative coding region is shown under the nucleotide sequence. Asterisks indicate the in-frame stop codons. Vertical arrowhead indicates the start site of the AR301 cDNA. The DDBJ accession number of this cDNA is D86122.

apparent difference between the spore formation efficiency of the truncated AR301 cDNA and the full length AML1 cDNA.

To determine whether AML1 can function as Mei2 protein, we examined the ability of AML1 to rescue the meiosis deficiency of *S. pombe mei2*$^-$ mutant. The pAH27 DNA was introduced into the *mei2*$^-$ disrupted mutant. AML1 cDNA could not rescue the meiosis deficiency of *mei2*$^-$ (data not shown) although AML1 cDNA could induce meiosis in RL190 cells as shown in the previous section. We obtained the same results using a inducible *nmt1* promoter. Thus, we concluded that the over-production of AML1 could not rescue *S. pombe* Mei2 protein deficiency. Mei2 is presumed to have dual function in meiosis, one is necessary for the entry into meiosis (premeiotic DNA synthesis) and the other is for the progression of meiosis (execution of meiosis I). A temperature sensitive mutant, *mei2-33*, is defective only in the execution of meiosis I at the semirestrictive temperature [3]. AML1 could not induce meiosis in *mei2-33* mutant cells at the semirestrictive temperature either (data not shown).

3.4. All plant organs contain AML1 transcripts

The expression of the *S. pombe mei2* gene is barely detected in normally grown cells. However, this gene is activated strongly under certain conditions that induce meiosis [2]. We would predict that the *AML1* gene is expressed only in gametic cell lines of higher plants. To examine this, we performed an organ-specific Northern blot analysis for the *AML1* gene. Surprisingly, as shown in Fig. 3, a strong hybridization signal at about 3.8 kb was observed not only in flowers but also in roots and shoots, and a weak signal was detected in siliques and in leaves. This result suggests that *AML1* is expressed in all organs and that the functions of AML1 protein might be necessary for all organs.

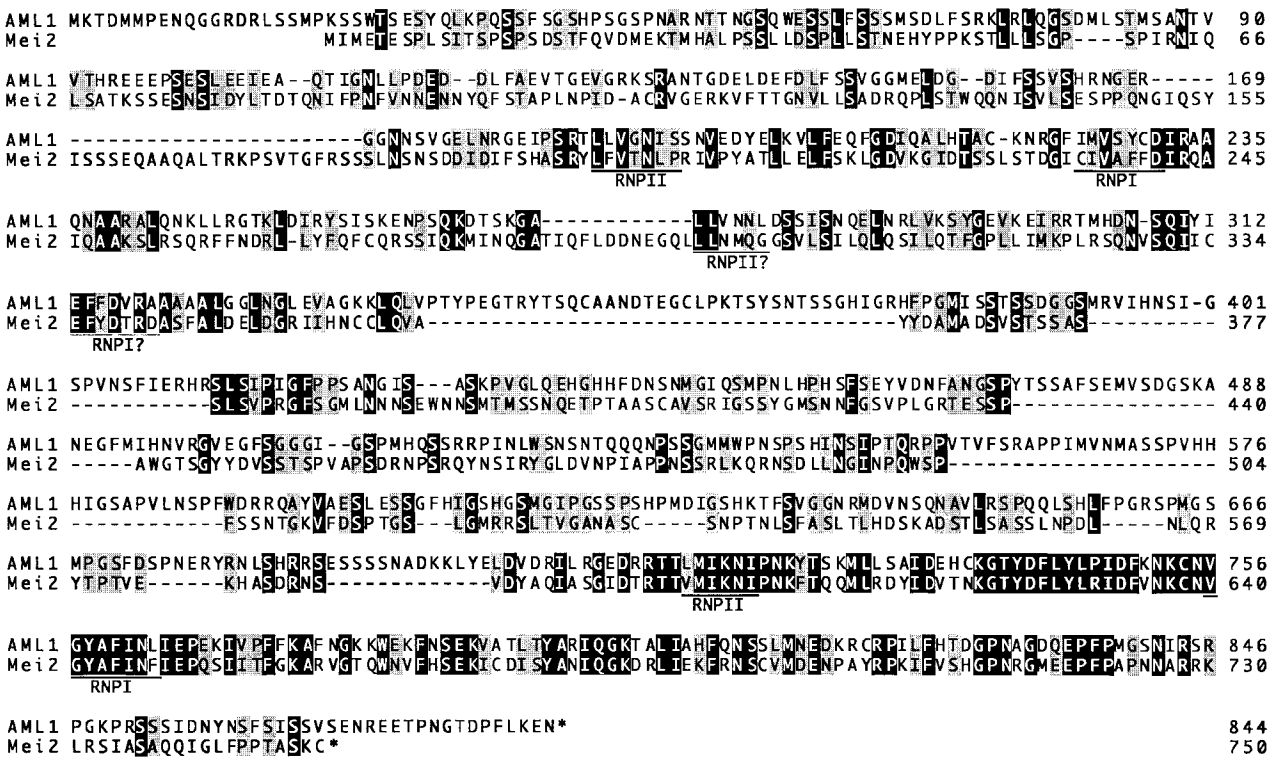

Fig. 2. Comparison of the amino acid sequence between AML1 and *S. pombe* Mei2 protein. Reversed characters and shaded characters indicate identical amino acid residues and conservative amino acids changes between two proteins, respectively.

3.5. Southern blot analysis of the AML1 gene

To learn whether *Arabidopsis* has other AML1-like genes or not, we performed genomic Southern blot analysis of *AML1* using the AML1 cDNA as a probe (Fig. 4). Under high stringency conditions, strong signals were obtained in *Eco*RI, *Hin*dIII or *Pst*I digests. Under low stringency conditions, an additional signal was observed in each restriction digest. These results indicate that an additional *AML1*-related gene exists in the *Arabidopsis* genome. Indeed, other two *mei2*-like cDNAs were found in *Arabidopsis* expressed sequence tags (40B12T7; 375 nt, and 140J21T7; 287 nt, 74% and 62% identity to *AML1* at nucleotide level, respectively) [21]. Thus, *Arabidopsis* has at least three *mei2*-like genes.

4. Discussion

In this study, we describe a novel cDNA encoding a Mei2-like protein, AML1, from the higher plant *Arabidopsis thaliana*, which was cloned by trans-complementation screening using a *S. pombe* pheromone receptor deficient mutant. The amino acid sequence of AML1 has significant similarity to that of *S. pombe* Mei2 protein. Mei2 is a key protein in the regulation of meiosis in fission yeast. Excessive expression of *mei2* can induce ectopic meiosis in a haploid cell (Y. Watanabe and Yamamoto, unpublished data). Thus, over-production of AML1 protein could induce meiosis bypassing pheromone signaling, similar to *S. pombe* Mei2 protein. However, AML1 cannot complement *mei2* null mutation. The spore formation efficiency of the truncated cDNA and the full length cDNA did not differ from each other, indicating that the C-terminal region of AML1 is sufficient to induce meiosis when it is over-produced in yeast cells. Furthermore, the high similarity between AML1 and Mei2 is restricted to their C-terminal regions. *S. pombe* Mei2 is necessary for both the premeiotic DNA synthesis and the execution of meiosis I, and its RNA binding activity in the C-terminal region is implicated in the earlier function of Mei2. [3]. Watanabe et al. have revealed that, besides the C-terminal region, the region between the second and the third RRMs (aa 429–532), whose amino acid sequence is not highly conserved in AML1, is essential for *S. pombe* Mei2 function [4]. Taken together, it is likely that AML1 can share with Mei2 only earlier function, which involves the conserved C-terminus structure and is re-

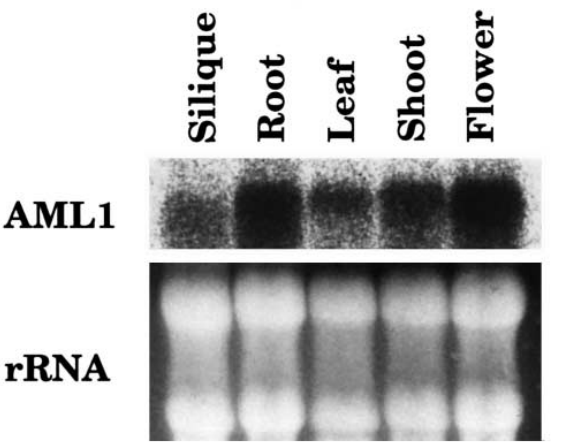

Fig. 3. Northern blot analysis of *AML1*. AML1 RNA levels in organs were examined by Northern blot analysis. A 30 μg total RNA was loaded per lane. The loaded rRNAs were visualized with ethidium bromide.

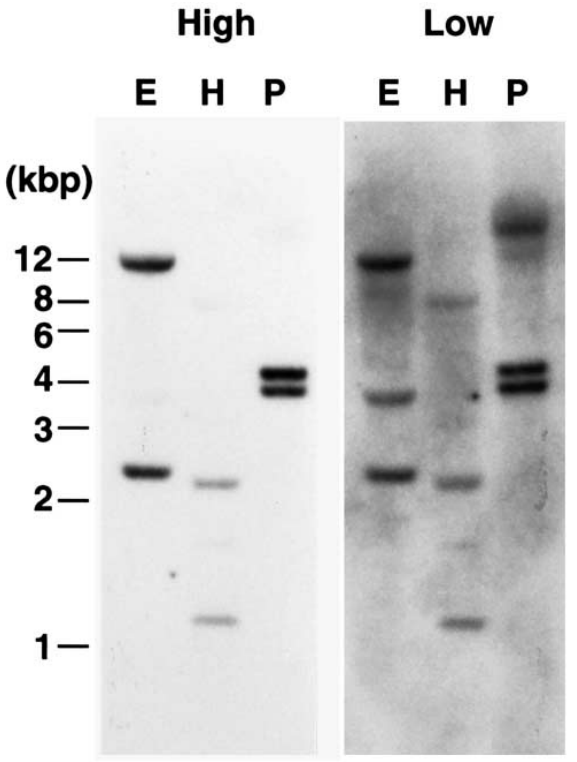

Fig. 4. Genomic Southern blot analysis of *AML1*. Total *Arabidopsis* DNA was digested with *Hin*dIII, *Eco*RI or *Pst*I and fractionated on a 0.7% agarose gel (5 μg/lane). High and Low indicate the autoradiograms obtained under high and low stringency conditions, respectively.

quired for the initiation of meiosis in fission yeast. This idea is supported by the results that AML1 could not suppress the sterile phenotype of *mei2-33* temperature sensitive mutant at the semirestrictive temperature. *mei2-33* is presumed defective only in the execution of meiosis I under such conditions [3]. *Arabidopsis* seems to have more than three *mei2*-like genes. Those gene products might share the functions of Mei2 protein. It might be of interest to examine whether the over-expression of those cDNAs in a cell can suppress *mei2*$^-$ mutation or not.

On the basis of similarity between Mei2 and AML1, and the effect of AML1 in *S. pombe* cells, we speculated that AML1 might be involved in the regulation of meiosis in higher plants. If this were the case, the *AML1* gene would be presumed to be transcribed preferentially in the gametic tissues. In contradiction to our expectation, *AML1* RNA was detected in almost all organs, although the transcription levels seemed different among them (Fig. 3). One possibility might be that AML1 is not required for meiosis but for other general cellular processes. However, it is noteworthy that the *S. pombe mei2* function is regulated at the post-translational level as well as at the transcriptional level [2,4]. Recently, Klucher et al. showed that the *AINTEGUMENTA* gene of *Arabidopsis*, whose product was required for ovule and female gametophyte development, was expressed in roots as well as in flowers [10]. Thus, we can expect that AML1 plays an important and specific role(s) in the commitment to meiosis in higher plants, as Mei2 does in fission yeast.

Although the mechanism by which Mei2 protein regulates the entry into meiosis is still unknown, its RNA binding activity is necessary for its functions. Our preliminary results showed that AML1 has also RNA binding activity (data not shown). To date, many RNA binding proteins from various organisms have been reported. RNA binding proteins are thought to control the expression of genes, by regulating transcription, RNA splicing, mRNA stability, efficiency of translation and transport of mRNA between nucleus and cytoplasm. Many of these proteins are implicated as developmental regulators in multicellular organisms [22,23]. Thus, it may be possible that AML1 function is not restricted to meiosis, but regulates various developmental processes.

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References

[1] Yamamoto, M. (1996) Trends Genet. 21, 18–22.
[2] Watanabe, Y., Iino, Y., Furuhata, K., Shimoda, C. and Yamamoto, M. (1988) EMBO J. 7, 761–767.
[3] Watanabe, Y. and Yamamoto, M. (1994) Cell 78, 487–498.
[4] Watanabe, Y., Shinozaki-Yabana, S., Chikashige, Y., Hiraoka, Y. and Yamamoto, M. (1997) Nature 386, 187–190.
[5] McCormick, S. (1993) Plant Cell 5, 1265–1275.
[6] Chaudhury, A.M. (1993) Plant Cell 5, 1277–1283.
[7] Preuss, D. (1995) Trends Genet. 11, 147–153.
[8] Robinson-Beers, K., Pruitt, R.E. and Gasser, C.S. (1992) Plant Cell 4, 1237–1249.
[9] Reiser, L. and Fischer, R.L. (1993) Plant Cell 5, 1291–1301.
[10] Klucher, K.M., Chow, H., Reiser, L. and Fischer, R.L. (1996) Plant Cell 8, 137–153.
[11] Elliott, R.C., Betzner, A.S., Huttner, E., Oakes, M.P., Tucker, W.Q.J., Gerentes, D., Perez, P. and Smyth, D.R. (1996) Plant Cell 8, 155–168.
[12] Sheridan, W.F., Avalkina, N.A., Shamrov, I.I., Batygina, T.B. and Golubovskaya, I.N. (1996) Genetics 142, 1009–1020.
[13] Kobayashi, T., Kobayashi, E., Sato, S., Hotta, Y., Miyajima, N., Tanaka, A. and Tabata, S. (1994) DNA Res. 1, 15–26.
[14] Nadeau, J.A., Zhang, X.S., Li, J. and O'Neill, S.D. (1996) Plant Cell 8, 213–329.
[15] Kitamura, K. and Shimoda, C. (1991) EMBO J. 10, 3743–3751.
[16] Tanaka, K., Davey, J., Imai, Y. and Yamamoto, M. (1993) Mol. Cell. Biol. 13, 80–88.
[17] Maundrell, K. (1990) J. Biol. Chem. 265, 10857–10864.
[18] Guthrie, C. and Fink, G.R. (1991) in: Method in Enzymology, Vol. 194, Academic Press Inc., San Diego, CA.
[19] Egel, R. and Egel-Mitani, M. (1974) Exp. Cell Res. 88, 127–134.
[20] Hirayama, T. and Oka, A. (1992) Plant Mol. Biol. 20, 653–662.
[21] Newman, T., de Bruijn, F.J., Green, P., Keegstra, K., Kende, H., McIntosh, L., Ohlrogge, J., Raikhel, N., Somerville, S., Thomashow, M., Retzel, E. and Somerville, C. (1994) Plant. Physiol. 106, 1241–1255.
[22] Bandziulis, R.J., Swason, M.S. and Dreyfuss, G. (1989) Genes Dev. 3, 431–437.
[23] Burd, C.G. and Dreyfuss, G. (1994) Science 265, 615–621.