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Molecular Characterization of an Anther-Preferential Gene from Rice

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Expression levels of anther-expressed genes in rice were estimated by plaque hybridization. A total of 33 cDNAs, isolated randomly from an anther-enriched cDNA library, were used as probes to hybridize both anther and leaf cDNAs. The expression level of individual cDNA clones was then estimated by counting the number of plaques hybridized to each probe. Based on abundance patterns that appeared in both anther and leaf cDNA libraries, the clones were classified into three groups. This classification showed that the majority of the clones (one group) exhibited expression in both cDNA libraries at almost equal frequency. The other two groups showed either low or no expression in the leaf cDNA library. Among the cDNA clones, *RA1003* (detected only in the anther cDNA library) was selected and further characterized at the molecular level. Consistent with the results of the plaque hybridization experiment, northern blot analysis also revealed no gene expression in vegetative organs, leaves, or roots. However, expression was high in the flowers, especially in the anthers. Detailed molecular studies of the gene are also described and discussed here.

Keywords: Anther-enriched cDNA library, anther-preferential gene, Rice

Male gametogenesis is a highly regulated developmental process that is essential to reproductive success in both plant and animal species. In flowering plants, reproductive processes take place within flowers containing microscopic male and female gametophytes that produce sperm and egg cells, respectively. The male gametophytes, i.e., pollen, complete their development within the sporophytic tissue of the anthers. Upon germination, each pollen grain produces two sperm cells that are transported into the embryo sac in the ovule to achieve double fertilization (McCormick, 1993).

A relatively large number of genes are required for the entire process of anther development. Approximately 10~20% of the genes are anther-specific, with the large majority being expressed in both the sporophyte and the male gametophyte (Willing and Mascarenhas, 1984; Willing et al., 1988). Functional analyses of anther-expressed genes have been conducted in many plant species. For example, the AtSUC1 gene in *Arabidopsis* encodes the sucrose symporter that is believed to be involved in anther dehiscence (Stadler et al., 1999). Recently, expressed sequence tag (EST) analysis using a tobacco anther cDNA library revealed that only 21 of the 200 cDNA clones randomly chosen from that library showed significant homology to the nucleotide sequences in the database (Choi and Hong, 2000). This suggests that, despite the characterization of many anther-expressed genes, a large number must still undergo functional analysis.

Pollen-expressed genes can be divided into at least two different groups, based on expression patterns. Those in the first group begin to express soon after meiosis, reaching their maximum by the late pollen interphase, and decreasing thereafter. The isolated *Brassica napus* cDNA clones, I3, E2, and Bp10, fall into this "early" gene category (Albani et al., 1990; Roberts et al., 1991; Foster et al., 1992). Genes in the second group start to express after microspore mitosis, with expression increasing until maturity. A few hundred different genes are estimated to be expressed very abundantly in mature pollen, comprising approximately a third of the total mRNAs (Willing et al., 1988). This expression pattern suggests a major function for these gene products during the latter part of pollen maturation and/or during pollen germination and tube growth. These "late" genes have been isolated from numerous plant species, including maize (Hanson et al., 1989; Turcich et al., 1993), tobacco (Weterings et al., 1992), petunia (Mu et al., 1994), tomato (Ray et al., 1988; Twell et al., 1989), sunflower (Baltz et al., 1992) and the Easter lily (Kim et al., 1994).

Only a few anther- or pollen-expressed genes have been isolated from rice, including two cDNA clones,

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YY1 and YY2, expressed in tapetal cells (Hihara et al., 1996). Another anther-specific gene, *RA8*, has also been reported as being expressed in both tapetum and pollen grains (Jeon et al., 1999). In the current study, we isolated several anther-expressed cDNA clones from an anther-enriched cDNA library and estimated their expression frequencies by plaque hybridization. Among these clones, an anther-preferential gene, *RA1003,* was studied in detail.

MATERIALS AND METHODS

Plant Materials and Bacterial Strains

For our molecular studies, rice plants (*Oryza sativa* L. cv. M201) were raised in a growth chamber at 26°C, under a 10.5-h-day cycle. *Escherichia coli* strains MC1000 [*F-,araD139, Δ(araABC-leu)7679, galU, galK, ΔlacX74, thi, rpsL(Str^r^)*] and JM83 [F-, *ara, Δ(lac-proAB), rpsL(Str^r^), ϕ80d lacZΔM15*] were used as the hosts for molecular cloning. The f1 helper phage R408 and *E. coli* strain XL-1Blue [F'::Tn*10 proA+B+, lacI^q^, lacZΔM15/recA1, endA1, gyrA96(Nal^r^), thi, hsdR17(rk-mk+), supE44, relA1, lac*] were used for in-vivo excision of the pBluescript plasmid vector from the λZapII phage (Stratagene, California).

Plaque Hybridization and Sequence Analysis

The rice cDNA library was constructed from mRNA of anther-enriched samples, prepared as described previously (Chung et al., 1994). Plaque hybridization was performed with 1×10^5 plaques lifted onto nitrocellulose membranes and hybridized with the radioactively labeled cDNA probes randomly selected from the anther-enriched cDNA library. After hybridization, the plaques on the nitrocellulose membranes containing either anther or leaf cDNA libraries were counted and grouped on the basis of plaque number. *RA1003* cDNA was sequenced by the dideoxy-nucleotide chain termination method, using double-stranded DNA as a template and T7 DNA polymerase (Pharmacia).

DNA and RNA Blot Analyses

Genomic DNA was prepared from two-week-old seedlings by the cetyltrimethylammonium bromide (CTAB) method. Eight μg of genomic DNA was digested overnight with 100 units of EcoRI, HindIII, and PstI restriction enzymes, then run on a 0.7% agarose gel. The digested DNA was blotted onto nylon membranes and hybridized with radioactively labeled probes. After hybridization, the genomic DNA blot was washed under highly stringent conditions (65°C). Total RNA was isolated according to the guanidium thiocyanate-CsCl method (Sambrook et al., 1989). Samples were harvested from flowers at the vacuolated-pollen stage. Anthers, carpels, and paleae/lemmas were dissected under a microscope and used in RNA preparations. Leaves and roots also were collected from two-week-old seedlings.

In-Situ Hybridization

In-situ hybridization experiments were performed by the method described previously (Chung et al., 1998). Flowers were embedded in either paraffin or LR White resin. These samples were then sliced and attached to glass slides. The tissue sections were hybridized with either antisense or sense RNA probes that were labeled with non-radio- or radioactive materials using digoxigenin-dUTP or α^{35}S-dUTP, respectively. Overnight hybridization was performed at 48°C in a humid chamber. Afterward, the slides were washed in 4× SSPE, 5 mM DTT for 5 min at 50°C; treated with 25 mg/mL RNase A in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 500 mM NaCl at 37°C for 30 min; and then washed in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 500 mM NaCl, and 5 mM DTT twice (15 min each time) at 37°C.

Molecular Genetic Mapping

The mapping procedure was basically the same as described by McCouch et al. (1988). While constructing the molecular genetic map, polymorphic DNA bands were detected by DNA gel-blot hybridization with two kinds of parental DNA (Nipponbare and Kasalath rices), each digested with eight different restriction enzymes: ApaI, BamHI, BglII, DraI, EcoRV, EcoRI, HindIII, and KpnI.

RESULTS AND DISCUSSION

Abundance Patterns of a Rice Anther-Enriched cDNA Library

To estimate the expression levels of rice anther cDNA clones, we performed a plaque hybridization experiment using rice anther and leaf cDNA libraries. A total of 33 independent clones, ranging from 0.6 to 1.5 kb, were randomly selected from a rice anther-

enriched cDNA library and used as probes. One hundred thousand plaques from each library were hybridized with the radioactive-labeled cDNA probes. By counting the number of plaques that hybridized to each probe, the expression level of individual cDNA clones was estimated (Table 1).

Based on their abundance patterns, the clones were classified into one of three groups: Group I included 21 cDNA clones that were present in both libraries at almost equal frequency. Within this group, the frequency varied between 0.001% and 0.9%. Group II contained six cDNA clones that showed preferential expression in the anther library and very little expression in the leaf library. The remaining six clones showed no hybridization to the leaf cDNAs and, therefore, were classified into Group III. This suggests that those six clones were probably highly abundant class genes in the pollen grains, with over 0.1% expression level, without expression in the leaves. We had previously observed similar results with lily pollen cDNA libraries (Kim et al., 1994a). This similarity in abundance patterns between lily and rice suggests that the plaque hybridization experiment was highly reliable.

Table 1. Abundance patterns of anther-expressed cDNA clones from rice.

Group	Clone	Anther (%)	Leaf (%)
I	RA201	0.020	0.010
	RA203	0.002	0.001
	RA204	0.006	0.004
	RA211	0.008	0.005
	RA215	0.900	0.400
	RA401	0.100	0.030
	RA404	0.018	0.030
	RA604	0.090	0.060
	RA605	0.196	0.096
	RA606	0.021	0.020
	RA607	0.002	0.003
	RA608	0.003	0.001
	RA610	0.006	0.002
	RA806	0.005	0.010
	RA807	0.014	0.008
	RA808	0.010	0.012
	RA809	0.001	0.006
	RA810	0.008	0.005
	RA1005	0.002	0.003
	RA1008	0.005	0.009
	RA1010	0.014	0.002
II	RA413	0.021	0.001
	RA609	0.146	0.014
	RA803	0.062	0.005
	RA813	0.090	0.039
	RA1001	0.061	0.003
	RA1009	0.039	0.008
III	RA8	1.280	0.000
	RA213	1.330	0.000
	RA415	0.220	0.000
	RA802	0.063	0.000
	RA812	0.016	0.000
	RA1003	0.100	0.000

Expression Pattern of the *RA1003* Gene

We previously reported that *RA8* was anther-specific and highly abundant in the anther library (Jeon et al., 1999). In the current study, another anther-preferential clone, *RA1003,* present at the 0.1% level, was selected for further investigation. The expression pattern for this gene was analyzed by RNA gel-blot hybridization using total RNA isolated from leaves and roots of two-week-old seedlings, as well as from different floral organs (Fig. 1). This experiment revealed that the gene was flower-specific, and was not detected in leaves or roots (Fig. 1A). The RNA blot hybridization showed that the gene was highly accumulated in the immature rice flowers, with the expression level decreasing in the later stages of floral development. In particular, the gene was expressed highly in the anthers and weakly in the paleae/lemmas and carpels

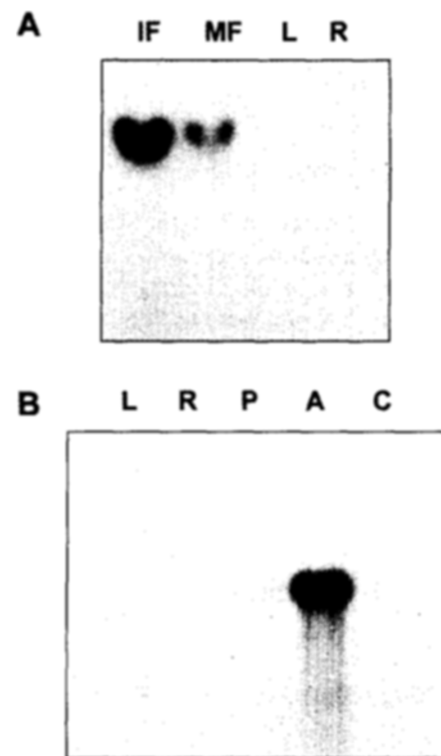

Figure 1. RNA gel-blot hybridization. Twenty-five μg of total RNAs isolated from each sample was used. **A.** *RA1003* mRNA expression in rice; IF, immature flower; MF, mature flower; L, leaf; R, root. **B.** *RA1003* mRNA expression in rice flowers; P, palea/lemma; A, anther; C, carpel.

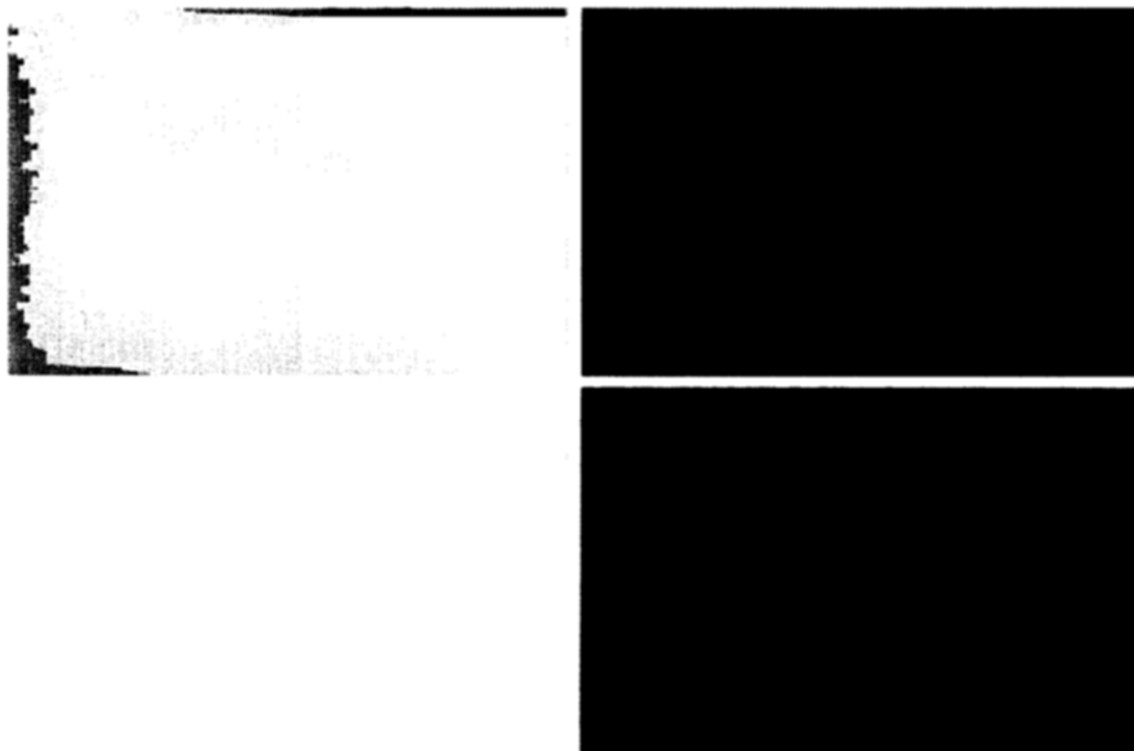

Figure 2. In-situ localization of *RA1003*. In-vitro transcript of *RA1003*, labeled with either digoxigenin-dUTP (A and B, antisense and sense probes, respectively) or α^{35}S-dUTP (C and D, antisense and sense probes, respectively), was hybridized to 8-μm sections of a paraffin-embedded immature flower (A and B) or 2-μm sections of a resin-embedded mature flower (C and D), respectively: a, anther; ip, immature pollen; mp, mature pollen; p, palea; t, tapetum. The scale bar represents 100 μm.

(Fig. 1B). These RNA gel-blot hybridizations suggest that the *RA1003* gene is anther-preferential, which is consistent with the results from the plaque hybridization (Table 1).

To further elucidate the expression pattern of the gene, especially in the anther, an in-situ hybridization experiment was conducted (Fig. 2). The *RA1003* transcript had high occurrence in the tapetum and pollen grains. Transcript was also weakly detectable in the inner layer of the palea and lemma. In the immature flower, expression was strong in the tapetum (Fig. 2A). However, in the mature flower, where the tapetum was degenerated, gene expression in the anther was restricted in the pollen grains (Fig. 2C). These results also demonstrate that *RA1003* is an anther-preferential gene in rice.

Sequence Analysis of the *RA1003* Gene

DNA sequence analysis revealed that the *RA1003* is 1344 bp long and contains an open reading frame of 358 amino acid residues (Fig. 3). This cDNA clone contained 76 nucleotides in the 5' untranslated region and 176 nucleotides in the 3' untranslated region. The frequency of G/C nucleotides at the third position of codons in the open reading frame was very high (60.7%) -- a common phenomenon in monocot plants. A search at the amino acid level showed weak homology to a family of glutelines, major storage proteins present in rice seeds (Okita et al., 1989; Kim et al., 1994b). In this family, Gluteline 1 was the most homologous to our RA1003 protein, with 27% identity and 50.6% similarity (data not shown). The RA1003 also showed 26.3% identity and 49.3% similarity to Gluteline 2, and 25.5% identity and 48.2% similarity to Gluteline 3. The RA1003 protein, however, did not contain a signal peptide, which is commonly present in seed storage proteins and is involved in targeting the proteins into storage. Despite the sequence homology with genes from the gluteline family, the role of RA1003 remains to be revealed.

DNA Gel-Blot Analysis and Molecular Genetic Map Position of the *RA1003* Gene

DNA gel-blot hybridization was used to study the

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1     CCCCAAATCCTCCTCATCTCCTCTTCCAGAACCTTTCTTTTTCGATTTCGTGTTCGAGTT
61    CTTTGTGATTGCAGCGATGGCGTCCGTGGATCTGACCCCGAGGCAGGCGAGGAAGGCGTA
                        M  A  S  V  D  L  T  P  R  Q  A  R  K  A  Y    15
121   CGGCGGCGATGGTGGGACCTACTACGAGTGGAGCCCCGCAGACCTGCCCATGCTGGAACT
       G  G  D  G  G  T  Y  Y  E  W  S  P  A  D  L  P  M  L  E  L    35
181   CGCCAACATCGGCGGCGCCAAGCTGTCGCTCAACGCCGGTGGCCTCGCCCTGCCCAGCTT
       A  N  I  G  G  A  K  L  S  L  N  A  G  G  L  A  L  P  S  F    55
241   TTCCGACTCCGGAAAGGTTGCGTATGTTCTTCAAGGCAAGGGCACTTGTGGCATTGTCCT
       S  D  S  G  K  V  A  Y  V  L  Q  G  K  G  T  C  G  I  V  L    75
301   GCCGGAGGCGAGCAAGGAGAAGGTGATCGCCGTGAAGGAGGGAGACTCCCTGGCACTCCC
       P  E  A  S  K  E  K  V  I  A  V  K  E  G  D  S  L  A  L  P    95
361   CTTTGGCGTGGTGACATGGTGGCATAACCTCCCGGAGTCCCCAATCGAGCTCGTCATCCT
       F  G  V  V  T  W  W  H  N  L  P  E  S  P  I  E  L  V  I  L   115
421   CTTCCTCGGCGACACATCGAAGGCGCACAAGGCCGGCCAATTCACAAACATGCAGCTCAC
       F  L  G  D  T  S  K  A  H  K  A  G  Q  F  T  N  M  Q  L  T   135
481   CGGTGCCACCGGCATCTTCACCGGCTTCTCCACGGAGTTCGTCGGCCGCGCATGGGACCT
       G  A  T  G  I  F  T  G  F  S  T  E  F  V  G  R  A  W  D  L   155
541   CGCCGAGTCCGACGCCGTCAAGCTCGTGTCCAGCCAGCCTGCCTCCGGCATCGTCAAGAT
       A  E  S  D  A  V  K  L  V  S  S  Q  P  A  S  G  I  V  K  I   175
601   CAAGTCCGGCCAGAAGCTCCCCGAGCCGTCGGCCGCCGACCGCGAGGGCATGGCGCTCAA
       K  S  G  Q  K  L  P  E  P  S  A  A  D  R  E  G  M  A  L  N   195
661   CTGCCTGGAGGCGCCGCTCGACGTGGACATCAAGAACGGCGGCCGCGTGGTGGTGCTCAA
       C  L  E  A  P  L  D  V  D  I  K  N  G  G  R  V  V  V  L  N   215
721   CACGGCGAACCTGCCGATGGTGAAGGAGGTCGGGCTCGGCGCCGACCTGGTGAGGATCGA
       T  A  N  L  P  M  V  K  E  V  G  L  G  A  D  L  V  R  I  D   235
781   CGGCCACTCCATGTGCTCGCCGGGGTTCTCGTGCGACTCGGCGTACCAGGTCACCTACTT
       G  H  S  M  C  S  P  G  F  S  C  D  S  A  Y  Q  V  T  Y  F   255
841   CATCCGGGCAGCGGCCGCGTCCAGGTGGTCGGCGCCGACGGGAAGCGCGTGCTGGACACC
       I  R  A  A  A  A  S  R  W  S  A  P  T  G  S  A  C  W  T  P   275
901   CACGTCGAGGGCGGCAACCTGTTCATCGTGCCGCGCTTCTGCGTCGTCTCCAAGATCGCC
       T  S  R  A  A  T  C  S  S  C  R  A  S  A  S  S  P  R  S  P   295
961   GACCTCCGGCCTGCAGTGGTTCTTCATTATCACCACACCAAACCCGATCTTCAGTCACCT
       T  S  G  L  Q  W  F  F  I  I  T  T  P  N  P  I  F  S  H  L   315
1021  GGCGGGGAAGACGTCGGTGTGGAAGGCGATCTCGCCGGAGGTGCTGGAGGCGTCGTTCAA
       A  G  K  T  S  V  W  K  A  I  S  P  E  V  L  E  A  S  F  N   335
1081  CGCGACGCCGGAGATGGAGAAGCTGTTCCGGTCCAAAAGGATCGACTCGGAGATCTTCTT
       A  T  P  E  M  E  K  L  F  R  S  K  R  I  D  S  E  I  F  F   355
1141  CGCGCCCAACTGAGCAGCTTCCCTTTGCCATTGCGTCGTCGCGTCGGTGTCGTCTTAAAT
       A  P  N                                                      358
1201  TAGGATTAAGTCATTAATAATTTCATCTCTTATCATGAGATGTTACTACCTTCTGTCAGT
1261  CTTATGCTATTGCGGTTGAACTGTGGTGATTCGCCACTTAAAATTCTATTCTATTCGAGA
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Figure 3. Nucleotide sequence and deduced amino acid sequence of *RA1003*. Nucleotides and amino acid residues are numbered on the left and on the right, respectively.

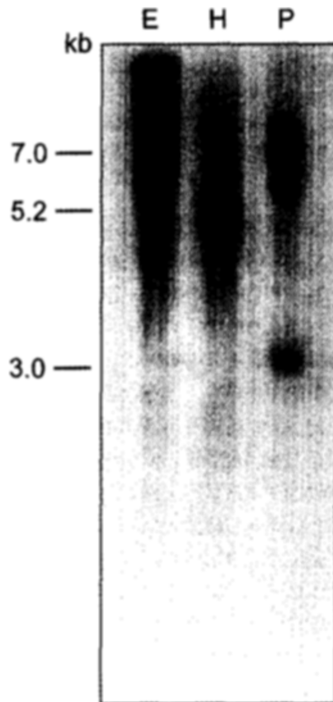

Figure 4. Genomic DNA blot analysis of *RA1003*. Eight μg of genomic DNA was digested with restriction endonucleases (EcoRI (E), HindIII (H) and PstI(P)), and hybridized with a full-length *RA1003* probe. The final wash was done at 65°C.

genomic organization of the *RA1003* gene in rice (Fig. 4). The probe hybridized to 2-3 DNA bands, suggesting that this gene forms a small family. It is still possible, however, that the gene belongs to a family of rice glutelin, another small gene family (Okita et al., 1989; Kim et al., 1994b). Map position of the *RA1003* gene was also determined by molecular mapping, using genomic DNA isolated from the F2 population of Nipponbare and Kasalath rices (Fig. 5). The map position was in the upper region of Chromosome 5 in which *RA1003*, designated as V12319, is 2 cM away from *R830* (Kurata et al., 1994).

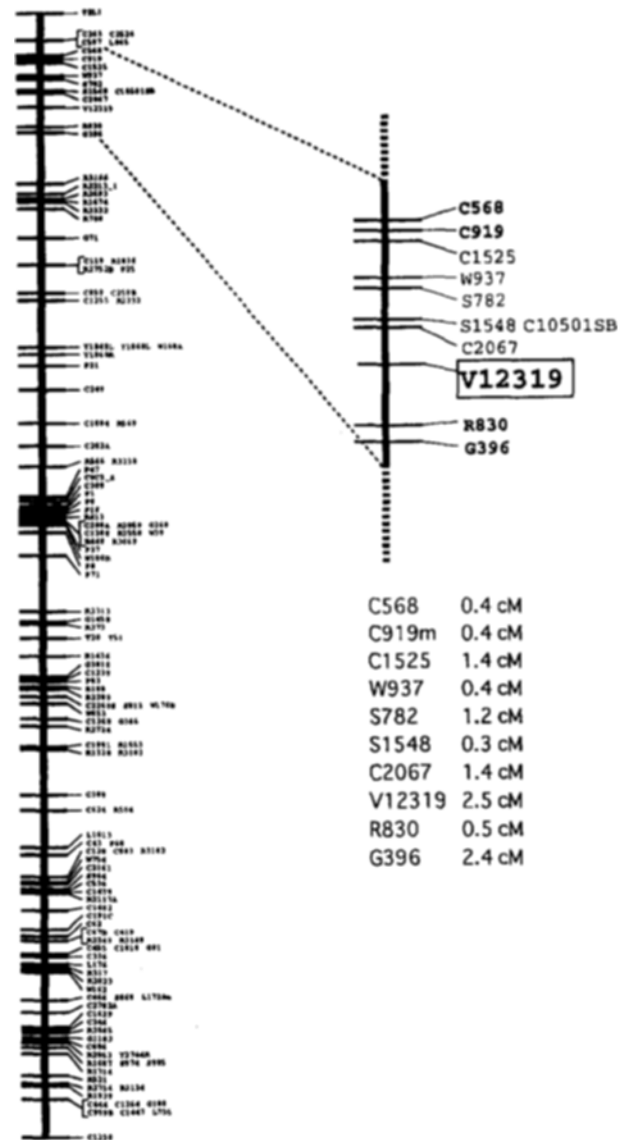

Figure 5. Molecular genetic map position of the *RA1003* gene, indicated as V12319 on Chromosome 5.

Although we have demonstrated through sequence homology and DNA blot analysis that this gene in rice may possibly act as a storage protein, i.e., glutelin, fur-

ther analysis will be necessary to conclusively reveal the function of RA1003.

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