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Sequence-tagged sites (STSs) as standard landmarkers in the rice genome

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Abstract Generating sequence-tagged sites (STSs) is a prerequisite to convert a genetic map to a physical map. With the help of sequence information from these STSs one can also isolate specific genes. For these purposes, we have designed PCR primer sets, of 20 bases each, by reference to sequences of restriction fragment length polymorphism (RFLP) landmarkers consisting of rice genomic clones. These markers were evenly distributed over the 12 chromosomes and were shown to be single copy by Southern-blot analysis. With improved PCR protocols, 63 standard STS landmarkers in the rice genome were generated. Similarity searches of all partial sequences of RFLP landmarkers by the FASTA algorithm showed that 2 of the 63 RFLP landmarkers, G357 and G385, contained part of the ORFs of aspartate aminotransferase and protein kinase, respectively.

Key words STS · RFLP · Rice · Genetic map
Coding region

Introduction

In a genome project, conversion of the genetic map to a physical map can be greatly accelerated by using sequence-tagged sites (STS) (Olson et al. 1989). An STS is a short stretch of genomic sequence that can be detected by the polymerase chain reaction (PCR) (Saiki et al. 1985). Each STS is mapped to a specified site as a landmark in the genome. In human and mammal genome research, a large number of STS primers have been produced by analyzing RFLP probe markers (Tang et al. 1992), microsatellites (Dietrich et al. 1992; Weissenbach et al. 1992), *Alu* elements (Nelson et al. 1989), expressed sequences (Durkin et al. 1992), end fragments of yeast artificial chromosome (YAC) inserts (Kere et al. 1992), and end sequences of cosmid clone inserts (Miwa et al. 1993). Utilizing these STSs for screening, e.g., yeast artificial chromosome (YAC) libraries, facilitates the construction of long contigs of physical mapping (Chumakov et al. 1992).

In plants, cDNA clones of *Arabidopsis* (Hauge et al. 1993; McGrath et al. 1993), maize (Gardiner et al. 1993), barley (Kleinhofs et al. 1993) and other plants (Tanksley et al. 1992 a, b) have been used as RFLP probes. Though few STS primers have been designed, there are methods in which STS primers are produced by using random amplified polymorphic DNA (RAPD) from the lettuce genome (Paran and Michelmore 1993) and by using RFLP of an STS product from the barley genome (Tragoonrung et al. 1992). In rice, many RFLP markers have been produced (McCouch et al. 1988; Saito et al. 1991; Tanksley et al. 1992 a). Williams et al. (1991) designed six STS primers from RFLP probe sequences, while Zhao and Kochert (1992) produced 11 STS primers using microsatellites. In order to develop standard STS landmarkers distributed evenly in all rice chromosomes, we selected random genomic clones which show single-copy RFLP probes. After partial sequencing of the RFLP probes, 20-base long-PCR primer sets were designed and used to establish 63 STSs.

Materials and methods

The 63 landmarker probes of the rice linkage map (Fig. 1) were derived from a previous map constructed from a cross of cultivars FL134 and Kasalath (Saito et al. 1991). The previous registration, XNpb***, was renamed G***. The probes were sequenced by the dideoxy termination method using the universal or reverse fluorescence dye primer with an Applied Biosystems automated DNA sequencer model 373A. Twenty-base-long oligonucleotide primers were chosen from each sequence with the aid of the commercial software OLIGO (version 4, National Biosciences). The oligonucleotides were synthesized by Applied Biosystems DNA synthesizer model 380B or 394A. PCRs were performed with the Cetus thermal

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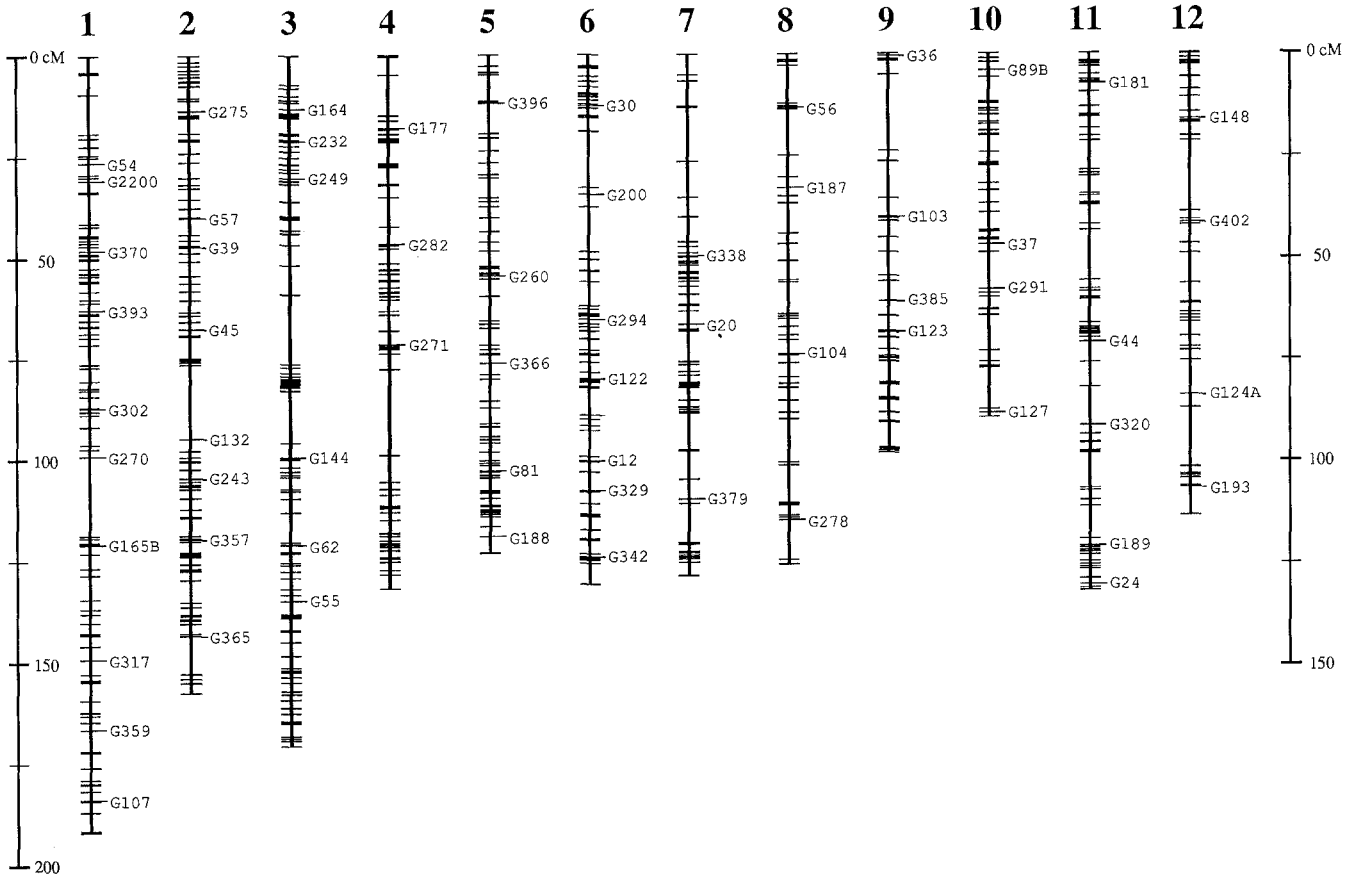


Fig. 1 The loci of 63 RFLP landmarkers in the rice genome. The rest of the markers are from Kurata et. al (in preparation)

cycler PL2000 or the MJ RESEARCH Programmable Thermal Controller PTC100. The template DNA was extracted from green leaves of the *japonica* variety Nipponbare by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). The 20-µl reaction mixture contained 400 nM of each primer, 200 µM of a dNTP mixture, 2.5 mM of $MgCl_2$, 20–30 ng of template DNA, 1 unit of *Taq* DNA polymerase (Promega) and 2 µl of 10× reaction buffer (Promega). In almost all reactions, repeated denaturation was at 94°C for 1 min, annealing at 55 or 60°C for 2 min, and polymerization at 72°C for 3 min. In cases with a low reaction yield, the number of PCR cycles was increased. If multiple reaction products were observed, the annealing temperature was raised (Rychlik et al. 1990). When multiple bands did not disappear, the magnesium ion concentration in the PCR reaction mixture was lowered (Love et al. 1990). The reaction products were electrophoresed on either 1.5% agarose or 3% NuSieve (3:1). DNA bands were stained by ethidium bromide and detected under UV light. A primer set that gave a single band by PCR was recognized as a unique primer set for producing a STS on a rice chromosome. Sequence similarity searches were done by DNASIS software and protein motif searches by MacPattern version 2.1 (Fuchs 1991) and PROSITE database (Bairoch 1992).

Results and discussion

In all, 63 designed primer sets gave a single band (Fig. 2). Each amplified fragment using total genomic DNA as a template was of the same size as the amplified fragment from the corresponding plasmid DNA. This means that the expected STSs regions were correctly amplified by PCR. The length of STSs ranged from 100 to 2000 base pairs. Some short fragments of less than 50 bp, considered to be primer dimers, were also observed.

Table 1 summarises the information for each STS. For G24, 39, 127, 132, 165B, 188, 193, 243, 357 and 366, the amplification yield was improved by increasing the number of PCR cycles from 30 to 35 or 40. For G30, 55, 103, 122, 177, 193 and 249, minor amplification products disappeared by setting the annealing temperature to 62, 65 or 70°C. Only G57, 165B, 370, 357 needed a change in the Mg^{2+} concentration to 1.0–1.5 mM to obtain a single band, as shown in Fig. 2.

For the first time, we succeeded in establishing 63 STSs from 63 landmarker sequences (100%). In contrast, in human genome research, a 83% and 84% efficiency has been reported (Weissenbach et al. 1992; Miwa et al. 1993). This may reflect the higher degree of nucleotide sequence repetitiveness in humans.

All the RFLP landmarkers, except clone G357, have been mapped by F_2 analysis. The Southern hybridization pattern of G357 was so smeared that we could not determine the locus. Nucleotide-sequence analysis of G357 revealed a TC repeat structure in this clone and this seemed to produce the smeared hybridization pattern. On the other hand, such a repeated sequence, or microsatellite, should be good for mapping because this structure can be highly polymorphic (Dietrich et al. 1992; Weissenbach et al. 1992; Zhao and Kochert 1992). We designed a primer set flanking the microsatellite observed in G357 (Fig. 3). When this primer set was used for amplifying Nipponbare and Kas-

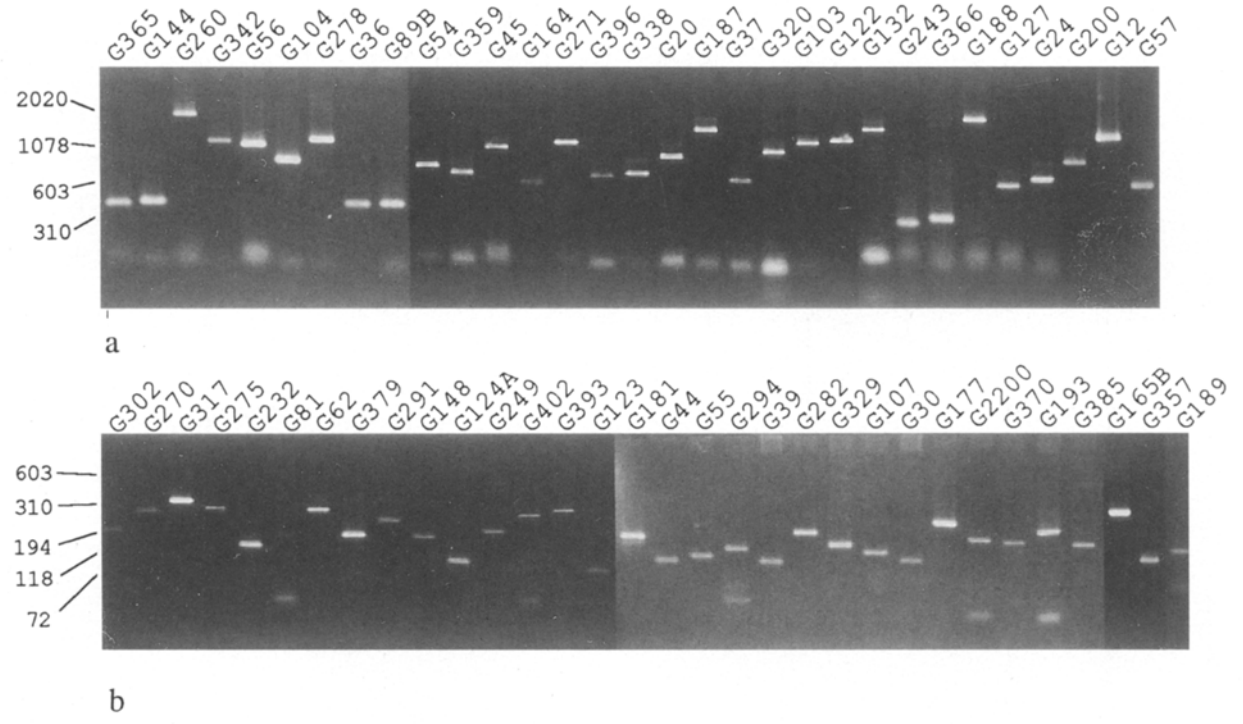


Fig. 2a, b Characterization of rice genome STSs. Ethidium bromide-stained 1.5% agarose (**a**), 3% NuSieve(3:1) agarose (**b**) gels containing PCR products amplified from Nipponbare total DNA. PCR conditions are described in Materials and methods and Table 1

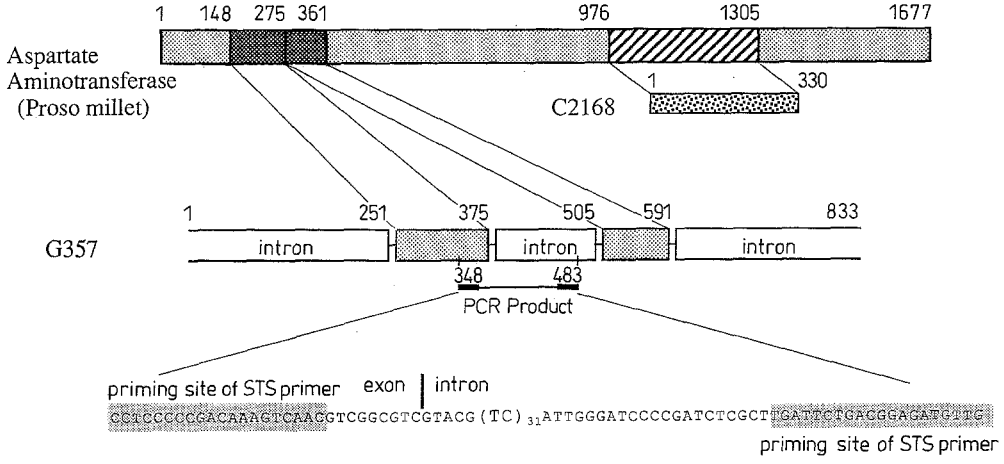


Fig. 3 A microsatellite in the G357 sequence and the relation between G357, C2168 and the aspartate aminotransferase of Proso millet

alath DNA as a template, a product length polymorphism was observed. This result enabled G357 to be mapped to chromosome 2.

Among the sequences from standard landmarkers, we found two plausible open reading frames (ORFs), using a similarity search by the FASTA algorithm. As shown in Figs. 3 and 4, part of the sequences of G357 and G385 contained ORFs of aspartate aminotransferase and protein kinase, respectively. As for G357, the sequences from nucleotides 252 to 375 and from 506 to 591 showed significant similarity to nucleotides 149 to 361 of the cDNA of aspartate aminotransferase from Proso millet (Taniguchi et al. 1992). Another cDNA clone, C2168 , from a rice callus cDNA library, also shows a sequence region similarity to the aspartate aminotransferase of Proso millet. However, the regions carrying nucleotide sequences similar to aspartate aminotransferase did not overlap with each other. A PCR with one primer in the G357 sequence (ATCTGGATCATGTGTAAAGC) and another in the C2168 sequence (CAGAAACTGCTTGCATCTCA) gave a single DNA band of about 2000 bp length using DNA from Nipponbare as a template (data not shown). In addition, G357

Table 1 List of RFLP marker sequences and STS primers

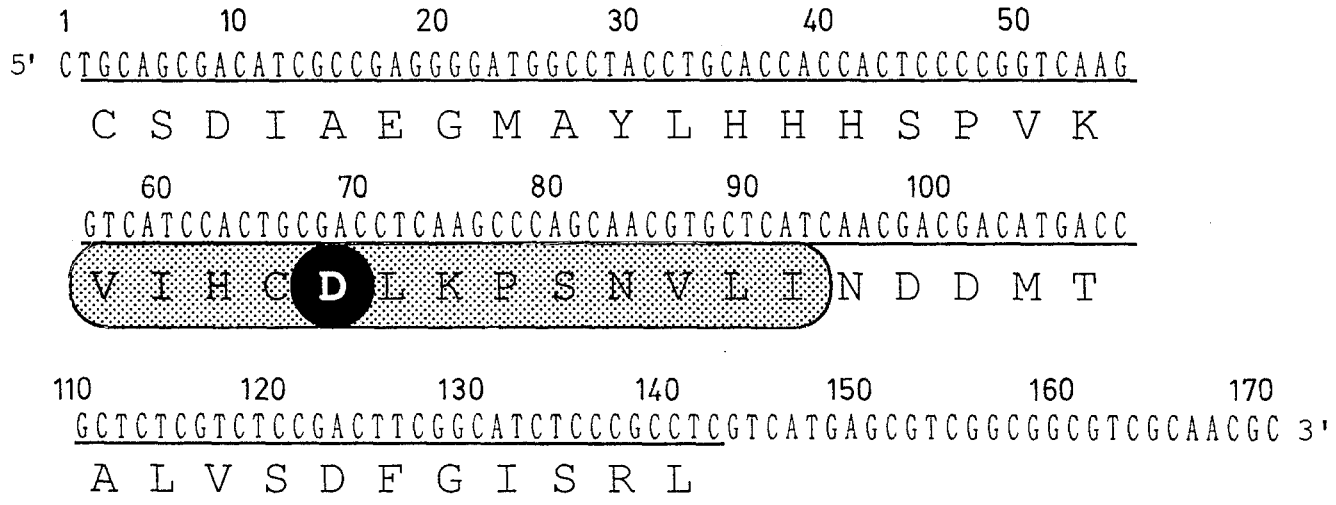
Chromosome no.	Clone name	STS primers 5′	STS primers 3′	Product length[b] (bp)	PCR condition: Annealing temp (°C)	PCR condition: Cycles	PCR condition: $MgCl_2$ (mM)	Accession no.[a]: For STS seq.	Accession no.[a]: For additional RFLP seq.
1	G54	ACATTAATGGAAGGCCCATG	TATGGTTCAGGAAGACAGAG	750	60	30	2.5	D13585 D13586	
	G2200	CAGATGACCGTCCGCTAACA	GCTCAACTCTTTGCTTTCTT	220	60	30	2.5	D14758 D14758	
	G370	AAGATGGCATTTGGTCAAGA	GTCGTCTGCTGCTGTTGCTG	208	60	30	1.0	D14788 D14788	
	G393	CAGAGAGGTGGCAGAGTTTT	GACAAGCGAAGGAGAAGAGG	294	60	30	2.5	D14794 D14794	D14795
	G302	GTCTCTCGCCACCGTCCTGA	AGGCACTTGAAATCCACTGC	180	55	30	2.5	D14778 D14778	D13532
	G270	GCACGGTGAGCCTCTTCAAG	TGTCTGTTTGTCCCTCTTCC	259	60	30	2.5	D14765 D14765	D14766
	G165B	CGCCGCCGACGAGCTATTCC	CCTGCGCCCACACCCTCACC	313	65	35	1.5	D14752 D14752	
	G317	GAGCCCAAGACGATTATCCA	AAAGCAATGTTACTGACCTA	312	60	30	2.5	D14781 D14781	D14782
	G359	TGGCACGAGGATCAGACATG	TGACATCTAGGAGTGGCAAC	600	60	30	2.5	D13587 D14787	
	G107	TCATCATCAAACTCATCGTC	AAGAACAACAACATCCTCAA	163	60	30	2.5	D14735 D14735	
2	G275	TTGCTTCCCTCCCATTAGAT	TACTGCCGATTTATTTGACG	271	60	30	2.5	D14768 D14768	D14769
	G57	CTCTTCACCTCCTCCACCTG	CGGCTCGCTCTTCTCCAACA	500	60	30	1.3	D14805 D14806	
	G39	CCGCCAATAATACAGTGATA	AAAACAAGCATAAAATACAT	136	60	35	2.5	D14796 D14796	D14797
	G45	TGGGATCAAAGTTGGCTATG	CGGAAACAGGGAAGCCTTTG	900	60	30	2.5	D13588 D13589	
	G132	GGCTACACACATGACACATG	AGCTCTTGCAACTTTAGTTG	1300	55	35	2.5	D13590 D13591	
	G243	ATTTACCAACTTAGGAATGC	CAGTATTGACAAGCAGGAAT	200	55	35	2.5	D13592 D13592	
	G357	CAACATCTCCGTCAGAATCA	CCTCCCCCGACAAAGTCAAC	137	60	40	1.0	D16340 D16340	
	G365	GTGTCACACTGTGCCTGATG	CGTGCCAAGATCAATCGCAG	350	55	30	2.5	D13537 D13537	
3	G164	CCAAATTGGCTGACCGATGA	TTCGAAGTGAAGAGATGGGA	580	60	30	2.5	D13528 D14751	
	G232	CTAGGCGAAGACTCCGATTC	TACCAAAAACTGAACCACAT	139	60	30	2.5	D13535 D13535	D13534
	G249	CAGGGGAAAGGGACCAAGCA	CGGCGGCGAGGCAGAACACG	204	70	30	2.5	D13531 D13531	D13530
	G144	GAGCTACTTAGTACGTAGCA	GGCATGCGATAGTCAAAGCA	376	55	30	2.5	D13527 D13527	D14745
	G62	TGAGCAGGGGCATAGAAACT	CAGAACAAAAACAACAACAC	276	60	30	2.5	D14808 D14808	D14807
	G55	CGTGTGATTACCCGCACTTT	CCCTACCGTGAGCCCCAACC	144	65	30	2.5	D14802 D14802	
4	G177	CAGGGGAAGAAATGGTGAGC	TGGGATGCGGAGGTAGGAAC	297	65	30	2.5	D14754 D14754	D14755
	G282	CAGCAGAGCACAGATACAAG	AGCAACATAAAGAAGTCACG	237	60	30	2.5	D14772 D14772	
	G271	TAGGAAAACAAGTTCACCGC	TGGGCCCCAAGTTCTACCTC	1100	60	30	2.5	D13595 D14767	
5	G396	TTCGCATTCTTGGCTGGTGT	GGTGATCTGGATGAATCTGA	600	60	30	2.5	D13596 D13597	
	G260	AGCAACTAAGCAAGAACTAC	AGTACATAGGGACAGAATTG	1700	55	30	2.5	D13533 D13598	
	G366	GAAGTATTGGTCAGGTCAAA	ACCCAGAGCAGCCTCCTTTA	300	55	35	2.5	D13599 D13599	
	G81	TCTTGCTCTGCTTCGTGCTG	GGCTATCACTCATTTGACAC	173	60	30	2.5	D13524 D13524	
	G188	AGAAAAAGCCAAGAAAGAAT	TCACCGTAAGAGATGTCAAC	1700	55	35	2.5	D13600 D13601	

[a] Accession numbers are for the partial sequences of RFLP landmarkers submitted to DDBJ. When we obtained a sequence from the same plasmid, the same accession number is shown for this clone. When we obtained two different sequences from different plasmids, two identical accession numbers are shown for this clone

Table 1 (continued)

Chromosome no.	Clone name	STS primers 5′	STS primers 3′	Product length[b] (bp)	PCR condition: Annealing temp (°C)	PCR condition: Cycles	PCR condition: $MgCl_2$ (mM)	Accession no.[a]: For STS seq.	Accession no.[a]: For additional RFLP seq.
6	G30	ATCCCTCACGCACTCCTTGT	GCCGCCGCCTACCTCCTCAT	141	65	30	2.5	D14779 D14779	D14780
	G200	TTCCGTTATGCCCAGTGATG	GGTATTATTCCCGACAAGTT	700	60	30	2.5	D14760 D13529	
	G294	GTCAGCACAACGACAACCTT	GGCAAGCTCAATCCCAAGTT	170	60	30	2.5	D14774 D14774	D14775
	G122	CACCATGACAGACCAAGCCA	CGGGGAGGAGTAACGAGAAG	1100	70	30	2.5	D14738 D13602	
	G12	CGGAAGATGCGCGAGGTAAC	GGCCATGTTTACTTAGGGAT	1100	55	30	2.5	D13461 D14744	
	G329	CACCGACCCACCGCCAAATG	AACAAAACCAAGAAATCACG	186	60	30	2.5	D14783 D14783	
	G342	GCAGTAAAACCCTGAAAAAT	CTGTCCTGTCTTAGTTGTTA	1100	55	30	2.5	D14786 D13603	
7	G338	AAGTGAGGGGGAGAAGAAAC	TTTCCTCAGCTAAGGGCATG	600	60	30	2.5	D13604 D13646	
	G20	CTCCAATTCTTTGATCGACA	AAGCACGAGAGGATGATTCT	850	60	30	2.5	D14761 D14762	
	G379	AGACGGTTGAGAGCACAGAT	AGGGGAAAGGAGTCGGTTCT	176	60	30	2.5	D14789 D14789	D14790
8	G56	CAACAGCTACTTCCTGAAAC	GGCAGGGATATTAGGACTAG	1000	55	30	2.5	D14803 D14804	
	G187	ACAAGGAAAGAGCTGAACAC	CAAGAAAGTCTTCTACGAGT	1400	60	30	2.5	D14757 D13605	
	G104	GCGTTGTGGAATATCCATTG	GGAAAGTCGACGTAACACAC	780	55	30	2.5	D13606 D13607	
	G278	GCACCTTCCTAGGATTACCT	GGCATTTCAAGGGTCCGTAC	1000	55	30	2.5	D14770 D14771	
9	G36	AGGCAATAGAACTTACCACT	CACGGGATGAAAAATACAGA	371	55	30	2.5	D13521 D13521	
	G103	TACCCTCCGAAGTAGCTAGC	TTGCGCTTGGCGACGGTCAC	1100	65	30	2.5	D13608 D14734	
	G385	GAGATAGGAAGGAAGAGCAT	GGAAGATTATTTGTCAGGAA	196	60	30	2.5	D14792 D14792	D14791
	G123	AGATTGTGCCAAAAAGAAAG	GTGTAAATGCTGCTTCCTCT	97	60	30	2.5	D14740 D14740	D14739
10	G89B	GCTCTTCCCAGCGTGTACAG	CATTGGAGAGAACTATGGTG	400	55	30	2.5	D13525 D13522	
	G37	CAAATGCTTGGGAGGGCCAT	GCAGTTCTTGGCAGGATAAT	580	60	30	2.5	D13523 D13523	
	G291	CTTTCTGGCAGTATTGTCCA	CCAACAGTAGAGTTCTCATT	233	60	30	2.5	D14773 D14773	
	G127	GGTTTTAGTATTCCTTATCT	GATACATCTCATCAGAATCA	550	55	35	2.5	D13526 D14743	
11	G181	CAGGGAAGGGAAGTTCACAT	ATAGCATTGAGTTACAGTTG	208	60	30	2.5	D14756 D14756	
	G44	GCTACGCAGACGCATAAAGA	GTCGCTCAGGTCCCCAAGAT	130	60	30	2.5	D14800 D14800	D14801
	G320	TTCCCAACCTGAAGACAATG	ATTTGTGGTACCATGCCATG	900	60	30	2.5	D13536 D13611	
	G189	GAATGTGATGAGAAGGTTGG	AAACTCAGACAACTCCTTGC	143	60	30	2.5	D21838 D21838	
	G24	TTCTGCTGATACTGACACTG	AACCTGTCCAAGACCATCTG	600	55	35	2.5	D13462 D13612	
12	G148	CAGTTTCAGTCCCATCTCCT	TTCGCTCAAGTTTACATCAA	177	60	30	2.5	D14746 D14746	D14747
	G402	AACTGAAGTGCTTGGTTTTG	TAAATGCGAAATCTGATACC	272	60	30	2.5	D14799 D14799	D14798
	G124A	TTGTGATTCAGAGGCAATGC	CCACCACCGATGGCTTCCTG	109	60	30	2.5	D14741 D14741	D14742
	G193	CATCAAGAAAGAGGAAGCAG	CAGCAGAACCACCCAAAACT	256	62	35	2.5	D14759 D14759	

[b] Product lengths of G12, 20, 24, 45, 54, 56, 57, 89B 103, 104, 122, 127, 132, 164, 187, 188, 200, 260, 271, 278, 320, 338, 342, 359 and 396 were measured by electrophoresis, and those of the rest were calculated from their sequences



a G385

Consensus Pattern : [LI**V**MFYC]-x-[**H**Y]-x-**D**-[**L**IVMFY]-**K**-x(2)-**N**-[**LIV**MFYC](3)

b [D is the active site residue]

Fig. 4 a, b The partial nucleotide sequence of G385 and the translated amino-acid sequence which showed similarity with protein kinase by the FASTA algorithm (**a**). The framed part of the amino-acid sequence corresponds to the protein kinase motif of the Prosite database (**b**)

showed the same locus as C2168 by F_2 analysis. This is strong evidence that G357 and C2168 are derived from the same gene coding for aspartate aminotransferase.

In the case of G385 (Accession No. D14791), the motif specifying protein kinase was found in its nucleotide sequence from 56 to 93 (Fig. 4). The amino-acid sequence of this motif contains the Asp residue essential for kinase activity also found in the G385 translated amino-acid sequence, indicating that clone G385 contains part of a protein kinase directing gene.

Based on the ratio of ORFs found in randomly-cloned genomic sequences, we can estimate the number of genes in the rice genome. Among the 36 kb sequenced, there was about 350 bp of coding region, that is, 210 bp of aspartate aminotransferase (Fig. 3) plus 140 bp of protein kinase (Fig. 4). The average ratio of finding known genes from public databases was estimated as 20% from a large-scale cDNA analysis of rice (our laboratory data). A combination of these data means that about 4.9% rice of genomic DNA contains coding regions. The genome size of rice is estimated to be 4.3×10^8 bp (Arumuganathan and Earle 1991). The average size of genes is estimated as 870 bp (=average amino-acid length in PIR database, 290, multiplied by three). Taking account of these values, we can calculate the number of genes within the rice genome to be 24000. This is a considerably less than the estimated number of genes, 100000, in the human genome (Singer and Berg 1991).

Construction of a physical map requires many STSs to screen YAC libraries. Sixty-three STSs based on genomic RFLP landmarkers were distributed in the rice genome of 4.3×10^8 bp, giving an average distance of 6.5×10^6 bp for each STS. The average insert length of the YAC library constructed in our laboratory was 3.5×10^5 bp. In order to cover all rice chromosomes by STSs targeted to YACs, about 1200 STSs are required. In our Rice Genome Research Program, we intend producing RFLP markers not only by random genomic DNA fragments but also by cDNAs (N. Kurata, in preparation). In future, the number of STSs could be easily increased using nucleotide sequences of cDNAs instead of RFLP markers.

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