Identification of a Seed Phospholipase D Null Allele in Rice (Oryza sativa L.) and Development of SNP Markers for Phospholipase D Deficiency

Yasuhiro Suzuki, Yoshinobu Takeuchi, and Kenta Shirasawa

ABSTRACT
Rice (Oryza sativa L.) bran usage is severely limited by the rapid degradation of rice bran oil and rapid development of hydrolytic rancidity. Because phospholipase D (PLD) disintegrates phospholipid membranes of oil bodies to deteriorate bran quality, we isolated a PLD-null rice mutant, ‘03-s108’, whose PLD-null phenotype is inherited as a single recessive trait. To develop a convenient DNA marker-assisted selection method for the PLD-null trait, we first performed a linkage analysis between the PLD-null phenotype and simple sequence repeat (SSR) markers by using F2 progeny from 03-s108 × ‘Koshihikari’ crosses, and we mapped the PLD-null genotype to chromosome 1 near a putative PLD gene sequence, PLDα1. DNA sequencing of the 03-s108 allele of PLDα1 revealed a transition mutation (G to A) in the third exon. This change resulted in a stop codon, indicating the presence of a nonsense mutation in the 03-s108 allele. Using this DNA sequence information, we then developed methods of cleaved amplified polymorphic sequence (CAPS) and dot-blot single-nucleotide polymorphism (SNP) detection for this mutation, and we confirmed that the sequence polymorphism was predictive of the presence or absence of the seed PLD-null phenotype. These CAPS and dot-blot-SNP methods are easy and cost-effective means for identifying seed PLD-null strains, to enable screening and breeding of PLD-null rice varieties.
PLD1-s108 DIG-CCTCGCTGATATGAGTC
PLD1-NB DIG-CCTCGCTGGTATGAGTC

Probes for dot-blot-SNP analysis of PLD1-2761R GCAGGTAAGCCCTCCCAATATTCG PLD1-SNP
PLD1-2503F CACGTGAGCTCATGTCAACAGTTTG PLD1-SNP

Primers for amplification in CAPS and dot-blot-SNP analysis of 13 RV3-2 CAACAACGCTAAACAGTAG PLD1-3
12 RV3 See above PLD1-3
11 FW3-3 TCTTCTGCTCTCTAAATCTG PLD1-3
10 FW3-2 CTGGCAAAGGAGAACAATG PLD1-3
9 FW3 See above PLD1-3
8 RV2-2 TGAACAATGCTGCCTGAG PLD1-2
7 RV2 See above PLD1-2
6 FW2-2 CTGTGTGTGATGTGTGCTT PLD1-2
5 FW2 See above PLD1-2
4 RV1-2 AAGAAGAAGGGGAGCAGA PLD1-1
3 RV1 See above PLD1-1
2 FW1 See above PLD1-1

Primers for sequencing of RV3 AACAGATGATGAATGCCATGT PLD1-3
FW3 GGGATGTTCTTTACAATTTCG PLD1-3
RV2 CTCAGGGGTATCAGGGAACC PLD1-2
RV1 AGAGCAAGAGCAAAGACGAGTA PLD1-1
FW1 GGTGTGAGGCTTCAAACCTAG PLD1-1

Table 1. Polymerase chain reaction primers and probes for dot-blot-SNP analyses.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)</th>
<th>Region†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers for amplification of RPLD1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FW1</td>
<td>GGTGTAGGCTCCTCAACATCG</td>
<td>PLD1-1</td>
</tr>
<tr>
<td>RV1</td>
<td>AGAGCAAAGACACAGAAGCAGT</td>
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</tr>
<tr>
<td>FW2</td>
<td>CGAGGAGGGGACCGCCTAATCGA</td>
<td>PLD1-2</td>
</tr>
<tr>
<td>RV2</td>
<td>CTCAGGGGTATCAGGGAACC</td>
<td>PLD1-2</td>
</tr>
<tr>
<td>FW3</td>
<td>GGGATGTTCTTTACAATTTCG</td>
<td>PLD1-3</td>
</tr>
<tr>
<td>RV3</td>
<td>AACAGATGATGAATGCCATGT</td>
<td>PLD1-3</td>
</tr>
</tbody>
</table>

| Primers for sequencing of RPLD1 | |
| 1 FW1 | See above PLD1-1 |
| 2 FW1-2 | CATTGTCATACATCAACT |
| 3 RV1 | See above PLD1-1 |
| 4 RV1-2 | AAGAGGAAGGGGAGCAGA |
| 5 FW2 | See above PLD1-2 |
| 6 FW2-2 | CTGTGTGTGATGTGTGCTT |
| 7 RV2 | See above PLD1-2 |
| 8 RV2-2 | TGAACAATGCTCCTGAG |
| 9 FW3 | See above PLD1-3 |
| 10 FW3-2 | CTGCGAAGAGGAACAAATGG |
| 11 FW3-3 | TCTTCTGCTCTAATCTCG |
| 12 RV3 | See above PLD1-3 |
| 13 RV3-2 | CAAACACGCTAAACAGTAG |

† See Fig. 1.

product. These results indicate that PLD acts as a trigger for the initiation of lipid decomposition and the subsequent deterioration of rice grain and rice bran fractions.

From the rice bran fraction, Ueki et al. (1995) purified a PLD protein (designated RPLD1, synonymous with PLDα1), determined several partial amino acid sequences, and produced anti-PLD polyclonal antibodies against the RPLD1 protein. With these antibodies, we identified a mutant line, ‘03-s108’, that lacked the PLD protein in seed, and showed by genetic analysis that the PLD-null characteristic is controlled by a recessive gene (Suzuki, 2011). Thus, it became possible to breed for reduced PLD activity in rice seeds, but screening for PLD-null plants required progeny testing of mature seeds with anti-PLD polyclonal antibodies (Ueki et al., 1995) and allowed them to set seed, then grew F2 plants and harvested F3 seeds from selfed F2 plants. The PLD protein level in seeds was determined as described previously (Suzuki, 2011) using anti-PLD polyclonal antibodies (Ueki et al., 1995). Genomic DNA was isolated from leaf by the method of Murray and Thompson (1980). Simple sequence repeat (SSR) markers were used for linkage analysis of PLD deficiency. The SSR analyses were performed as described by McCouch et al. (2002).

DNA Sequence Analysis

DNA sequence analysis was performed by direct sequencing of polymerase chain reaction (PCR) products. Since the PLD gene is quite large to amplify as a single fragment, we constructed three primer pairs that covered the entire PLD gene region and its putative promoter region (Table 1 and Fig. 1). The PCR mixture (10 μl) contained ~10 ng of plant DNA, 10 pmol of each primer, 1X ExTaq buffer, 2 nmol of each dNTP, and 0.5 U of Taq DNA polymerase (ExTaq, Takara Bio, Otsu, Japan). Thermal cycling conditions for amplification of PLD gene fragments were as follows: 1-min denaturation at 94°C; 35 cycles of 10-s denaturation at 98°C, followed by 1-min annealing and extension at 68°C; and a final 3-min extension at 72°C. The amplified DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan) after 1% agarose gel electrophoresis, and its sequences were determined by direct sequencing using a DNA sequencer (Applied Biosystems, Foster City, CA). The sequences of PLD were analyzed using RiceBLAST (http://RiceBLAST.

MATERIALS AND METHODS

Plant Materials

Four rice varieties and lines were used in this study. ‘Koshihikari’ and ‘Nipponbare’ are wild-type rice varieties that contain normal levels of PLD activity in seeds. ‘ND0052’ is a line in which transposon Tos17 is inserted into PLDδ2 (Os03g0840800; Miyao et al., 2003; Suzuki, 2011), a gene unrelated to the seed PLD-null phenotype. 03-s108, a mutant identified from a mutagenized population of ND0052 seeds, lacks PLD activity and protein in seeds (Suzuki, 2011).

Linkage Analysis and Genomic DNA Isolation

For linkage analysis of the seed-PLD-null allele, we crossed 03-s108 (null) with Koshihikari (normal). We grew the F1 plants and allowed them to set seed, then grew F2 plants and harvested F3 seeds from selfed F2 plants. The PLD protein level in seeds was determined as described previously (Suzuki, 2011) using anti-PLD polyclonal antibodies (Ueki et al., 1995). Genomic DNA was isolated from leaf by the method of Murray and Thompson (1980). Simple sequence repeat (SSR) markers were used for linkage analysis of PLD deficiency. The SSR analyses were performed as described by McCouch et al. (2002).
CAPS and Dot-Blot-SNP Analysis for Phospholipase D Genotyping

Single-nucleotide polymorphisms among Nipponbare, ND0052, and 03-s108 were identified by PLD gene sequence analysis. DNA fragments containing an SNP associated with the seed PLD phenotype (designated PLD1-SNP in Fig. 1) were amplified by PCR using the forward primer PLD1-2503F and the reverse primer PLD1-2761R (Table 1 and Fig. 1). Thermal cycling conditions were as follows: 2-min denaturation at 94°C; 35 cycles of 30-s denaturation at 94°C, 30-s annealing at 60°C, and 60-s extension at 72°C; and a final 10-min extension at 72°C. For CAPS analysis, the PCR products were digested with Pol (SibEnzymes, Novosibirsk, Russia). The digested DNA was subjected to electrophoresis on a 6% polyacrylamide gel in 0.5× TBE buffer. The resulting DNA bands were stained with ethidium bromide.

For dot-blot-SNP analysis, the PCR products were dot-blotted onto nylon membranes using a Multi-pin Blotter (Atto, Tokyo, Japan) (Shirasuresawa et al., 2006, 2008). The detection probes for the wild-type allele (found in Nipponbare and Koshihikari) and the 03-s108 allele were PLD-NB and PLD-s108, respectively, labeled with digoxigenin at the 5´ end of the oligonucleotide (Table 1). The probe mixture containing 25 nM of the digoxigenin-labeled oligonucleotide and 125 nM of unlabeled counterpart oligonucleotide was hybridized to the membrane overnight at 42°C. After hybridization, the membranes were washed twice in 0.5× SSC/0.1% SDS at 42°C for 20 min. Signals were detected with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate solution.

RESULTS

Linkage Analysis of Phospholipase D Deficiency by SSR Markers

Through genome-wide analysis, 17 PLD genes have been found in rice; five of the 17 PLD genes are PLDα-like genes (Li et al., 2007). These 17 genes are located on several rice chromosomes. The PLD protein purified from the rice bran fraction (designated RPLD1) is the product of one of the five PLDα-like genes, so the seed PLD that is deficient in 03-s108 is likely to be the product of one of these five genes, which are located on chromosomes 1, 5, and 6 (McGee et al., 2003; Li et al., 2007; Yamaguchi et al., 2009). We performed a homology search for PLD genes and found one putative PLD gene sequence (PLDα1) on chromosome 1. An SSR marker (RM3453) located near PLDα1 showed polymorphism between 03-s108 and Koshihikari. To ascertain whether the gene encoding the PLD-null character is located on chromosome 1, linkage analysis between the trait and the SSR marker was performed. Of 50 F2 plants, we found five recombinants between RM3453 and the PLD-null phenotype. The genetic distance between RM3453 and the locus controlling the PLD-null phenotype was calculated to be 5 cM. As there are no putative PLD genes on chromosome 1 except for PLDα1 (gene ID: Os01g0172400), these results indicate that the seed PLD that is deficient in 03-s108 is caused by a variation at the PLDα1 locus. Here, we designate the seed PLD-null allele of 03-s108 as pldα1-1.

DNA Sequence of pldα1-1

From the above data, we expected that pldα1-1 would contain mutations for the seed PLD-null phenotype; hence, we determined its nucleotide sequence. The Nipponbare allele of PLDα1, including the open reading frame and putative promoter region, is approximately 5.9 kb in size (Fig. 1). The nucleotide sequences of the ND0052 and 03-s108 PLDα1 alleles were determined and compared with the published Nipponbare sequence. Although the nucleotide sequences of PLDα1 in Nipponbare and ND0052 were completely identical, DNA polymorphisms between Nipponbare and 03-s108 were found in two regions. One polymorphism was a transition mutation from G (Nipponbare) to A (03-s108) in the second exon in region PLD1-2 (Fig. 1). In the open reading frame of the Nipponbare sequence, this G was the third nucleotide of the codon for tryptophan at position 82. In contrast, the A in 03-s108 produced the stop codon TGA, indicating that the translation of the predicted protein in 03-s108 would be terminated after the 81st amino acid. The other DNA polymorphism between Nipponbare and 03-s108 was a transition mutation from G (Nipponbare) to A (03-s108) in the second exon in region PLD1-1 (Fig. 1).
resulting in a substitution of alanine by threonine. As both amino acids have neutral characteristics and almost the same isoelectric point, this amino acid substitution might not have any measurable effect on the PLD-null phenotype. At the locations of these two SNPs between Nipponbare and 03-s108, the sequences of ND0052 and Koshihikari were the same as those of Nipponbare.

Detection of SNPs by CAPS and Dot-Blot-SNP Analysis

As described above, the SNP in the third exon of PLDα1 was the likely cause of the seed PLD-null phenotype in 03-s108 (Fig. 1). We utilized this information to develop easy and cost-effective methods for screening and breeding of PLD-null rice varieties. First, we focused attention on this SNP and its flanking sequences within PLDα1, for the purpose of developing rapid SNP detection methods. The flanking sequences in the Koshihikari and 03-s108 alleles were GTATGAGTC-GTTC and ATATGAGTCGTTC, respectively. Since the restriction enzyme PsI cleaves both sides of the nucleotide sequence (N)7GTANNNNNNGTTC(N)12, which is identical to the Koshihikari sequence, we used CAPS analysis to predict the seed-PLD-null phenotype. In CAPS analysis, the digested PCR products showed DNA fragments of different sizes in each genotype: 177, 145, 114, 82, and 32 bp in Koshihikari and ND0052, and 259 bp in 03-s108 (Fig. 2). We used the CAPS analysis to genotype 32 F2 plants and obtained the following results: nine plants were homozygous for the Koshihikari allele, 12 were homozygous for the 03-s108 allele, and 11 were heterozygous. For each F2 plant, the SNP genotype was completely consistent with the phenotype determined by western blot analysis, indicating the effectiveness of the CAPS analysis for identifying PLD-null plants.

Second, we applied dot-blot-SNP analysis to detecting SNPs predictive of the seed PLD phenotype. With the dot-blot-SNP method, SNPs in amplified DNA fragments on a nylon membrane can be detected by competitive hybridization with digoxigenin-labeled oligonucleotides having the sequence of one allele (e.g., the mutant allele pldα1-1) together with unlabeled oligonucleotides having the sequence of another allele (e.g., the wild-type PLDα1 allele). The PCR products amplified from the 44 F2 plants, Koshihikari, and 03-s108 DNA were dot-blotted onto duplicate nylon membranes. On the membrane hybridized with the labeled PLD-NB probe, positions dot-blotted with amplified PCR products from Nipponbare, a heterozygous control, and 31 F2 plants showed positive signals (Fig. 3). In contrast, on the membrane hybridized with the PLD-s108 probe, positions dot-blotted with amplified PCR products from 03-s108 DNA showed positive signals (Fig. 3).
from 03-s108, a heterozygous control, and 31 F₂ plants showed positive signals (Fig. 3). Positions in 18 of the 44 F₂ plants and a heterozygous control showed both Koshihikari and 03-s108 signals. Thus, the 44 F₂ plants were classified into 13 homozygous for the Koshihikari allele, 18 heterozygous, and 13 homozygous for the 03-s108 allele. These observed frequencies were not significantly different from a 1:2:1 ratio when tested with the chi-square goodness-of-fit test ($p = 0.483$). This finding is the same as that obtained in western blot analysis of PLD and in the CAPS analysis.

**DISCUSSION**

Triacylglycerols, the main lipids of rice bran, occur in oil bodies with phospholipid membranes that are disintegrated by PLD (List et al., 1992; Nakayama et al., 1981; Takano et al., 1989). Previously, we isolated a seed-PLD-null strain, 03-s108, which had no PLD enzymatic activities in seeds (Suzuki, 2011). Oil body membranes prepared from 03-s108 bran disintegrated more slowly than those from a PLD-normal variety (Suzuki, unpublished data, 2007). These results indicate that a PLD-null phenotype would be useful to maintain oil body membranes, but the widely available screening method for PLD-null strains is western blotting of progeny seed, which consumes considerable time and effort. Therefore, simple and easy screening methods are essential, and screening methods that utilize the nucleotide sequences responsible for PLD deficiency, as reported in this study, are the most informative.

Here, we used SSR markers to map the seed-PLD-null trait and found that it was located on chromosome 1. $PLD\alpha_1$ is the only putative PLD gene present on chromosome 1, providing strong evidence that the PLD-null allele in 03-s108 was an allele of $PLD\alpha_1$. By comparing Nipponbare and 03-s108 $PLD\alpha_1$ DNA sequences, a nonsense mutation was identified in the PLD-null strain (Fig. 1). With this information, we planned to develop an accurate and convenient PLD-null detection method. There are many methods for the analysis of SNPs, but most of these methods are not suitable for screening a large number of strains because of the need for expensive reagents or instruments (Hayashi et al., 2004; West et al., 2006). In the present study, we developed both CAPS (Fig. 2) and dot-blot-SNP analysis methods (Fig. 3) to detect the PLD-null genotype. The CAPS assay distinguishes between alleles in which an SNP results in a difference in restriction sites. The CAPS markers are codominant genetic markers, and many samples can be resolved on a polyacrylamide gel, depending on the apparatus size (Konieczny and Ausubel, 1993). Dot-blot-SNP analysis is a competitive hybridization technique (Shirasawa et al., 2006, 2008), and a large number of samples can be simultaneously analyzed using two small nylon membranes. Since the two methods developed in this report accurately detect the SNP corresponding to the PLD-null phenotype and do not require expensive reagents or instruments, they can serve as highly reliable, simple, and cost-effective methods for breeding and screening of a large number of strains with desired characteristics.

It should be noted that PLD-null characteristic of 03-s108 would be even more useful for improvement of rice bran, grain, and fat stability if combined with other related traits. Previously, we showed that LOX-3 is involved in the production of volatile compounds in stored rice and that the development of stale flavor is delayed in LOX-3-null rice (Suzuki et al., 1996, 1999). We identified the rice $LOX-3$ gene and developed SNP markers for the LOX-3 deficiency (Shirasawa et al., 2008). Recently, we identified PLD-null strain 03-s108 (Suzuki, 2011) and, in the present study, developed SNP markers for the PLD-null genotype. As described above, PLD serves as a trigger to initiate lipid degradation and the deterioration of bran quality, and LOX-3 peroxidizes unsaturated fatty acids, which are degradation products of TAGs. Therefore, combining the LOX-3-null and PLD-null traits may alleviate oxidative deterioration of rice grains and promote greater storage stability. This combination may also improve the fat quality and content of rice bran. To breed LOX-3 and PLD double-null strains, we are crossing seeds of LOX-3-null strains with 03-s108 in collaboration with the breeding groups at our institution (Suzuki et al., unpublished data, 2010). When selecting multiple SNPs simultaneously in a breeding program, such as LOX-3- and PLD-null, the dot-blot-SNP technique is an efficient method (Shiokai et al., 2010). Multiplex PCR is first performed with the primer pairs for each SNP, followed by dot-blot-SNP detection with different pairs of membranes for each SNP being detected.

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**References**


Devaiah, S.P., X. Pan, Y. Hong, M. Roth, R. Welti, and X. Wang. 2007. Enhancing seed quality and viability by suppressing phospholipase D in Arabidopsis. Plant J. 50:950–957. doi:10.1111/j.1365-313X.2007.03103.x