Relationship Between the First Base of the Donor Splice Site of Waxy Gene Intron 1 and Amylose Content in Yunnan Indigenous Rice Varieties

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Abstract: There exists a single nucleotide polymorphism, G or T, at the first base of the donor splice site of waxy gene intron 1 in rice. In order to study the relationship between the first base of the donor splice site of waxy gene intron 1 and amylose content in rice, the one-step PCR method was used to determine whether it is G or T in 220 Yunnan indigenous rice varieties from 14 districts, 55 towns/counties of Yunnan Province, and 101 varieties of which were validated by the PCR-Acc I method. According to the G/T polymorphism, 164 rice varieties showed GG-genotype, while the other 56 fell into TT-genotype, accounting for 74.5% and 25.5% of all the test varieties, respectively. When all the rice varieties were divided into indica and japonica subspecies, it was found that 80.5% of indica rice and 67.0% of japonica rice belonged to GG-genotype. The rice varieties with GG-genotype had significantly higher amylose content (18.95% on average) than those with TT-genotype, but 33 rice varieties with GG-genotype still had low amylose content ranging from 3.91% to 15.93%, and most of them came from the Dai minority area in the Southwest of Yunnan Province. However, there was no significant difference in the mean amylose content of the same GG or TT genotypes between indica and japonica rice, suggesting that different genetic backgrounds, indica or japonica, had no effect on amylose content. The coefficient of correlation between the genotype and amylose content was 0.733 (P<0.01).

Key words: Yunnan indigenous rice varieties; waxy gene; intron; the first base of donor splice site; amylose content; genotype; relationship

Amylose content is a key determinant of eating and processing qualities of rice [1-2]. When cooked, the rice with high amylose content is separate and less tender with good swelling capacity, while rice with low amylose content tends to be sticky and tender with less swelling capacity. With the development of standard of living, people have different demands for amylose content in terms of different purposes and uses. Thus, amylose content has been an important item to be considered in quality breeding of rice [3-4].

Amylose synthesis in rice is mostly controlled by the catalysis of granule-bound starch synthase (GBSS) encoded by waxy gene (Wx) [5-7]. The study of the expression regulation of waxy gene indicates that amylose content is determined by the splicing efficiency of waxy gene intron 1 [8]. The further studies showed whether the first nucleotide is G or T in the donor splice site of waxy gene intron 1 is related to the splicing efficiency of waxy gene intron 1 [9-13]. If the first base is natural G in the donor splice site, waxy gene intron 1 can be spliced normally and there will be more mature mRNAs from waxy gene and GBSS, thereby rice endosperm has high amylose content; contrarily if the first base G mutates into base T, waxy gene intron 1 will be spliced inefficiently by other splice sites and there will be few mature mRNAs from waxy gene and less GBSS, and then low amylose content in rice endosperm. Ayres et al [14] firstly designed PCR-Acc I detection method according to G/T SNP (single nucleotide polymorphism) at position +1 of the donor splice site of waxy gene intron 1, that is the base G at position +1 of the donor splice site of waxy gene intron 1, that is the base G at position +1 of the donor splice site of waxy gene intron 1 together with its flanking sequences can constitute Acc I recognition site AGGTATA on which the donor splice site of waxy gene intron 1 will be digested by Acc I; whereas other donor splice sites of waxy gene intron 1 including AGTTATA will not be. Through detection of 89 rice varieties, it was found that the high amylose content rice has base G at position +1 of the donor splice site of waxy gene intron 1 and can be digested by Acc I, while the low amylose content rice has base T and can
not be digested. On the basis of the above analysis method, Cai et al [15] improved PCR-Acc I marker and observed the amylose content in indica rice with the marker. Following this, based on the fact that the matching degree between 3’ terminal base of the primer and its template will make a great impact on PCR amplification efficiency, Mao et al [16] designed a one-step PCR method, which could also be used to detect G/T SNP at position +1 of the donor splice site of waxy gene intron 1, offering a simple, rapid and economical approach for bulk detection in breeding.

Yunnan Province of China is one of the centers with the most genetic and ecological diversity of rice in the world [17-19]. In this paper, we reported the detection result of the first base of the donor splice site of waxy gene intron 1 in 220 Yunnan indigenous rice varieties so as to reveal the relationship between the base G/T at position +1 of the donor splice site of waxy gene intron 1 and amylose content in rice.

MATERIALS AND METHODS

Plant materials

Two hundred and twenty rice accessions were used in the experiment, which were Yunnan indigenous rice varieties from 14 districts, 55 towns/ counties of Yunnan Province, China, and all were pure lines by self-pollination for many years. According to Cheng’s index method [20-21], 123 accessions were indica and 97 were japonica rice. These indigenous rice varieties almost distributed over the whole Yunnan Province and were located in quite diversified climatic ecotypes.

All 220 rice materials were grown at Yaojie town, Xinping County, Yunnan Province (altitude 500 m) in 2003. One hundred plants for each variety were planted, and harvested after ripening. The managements followed the common method in paddy fields.

Amylose content measurement

The amylose content of rice was determined according to China National Standard GB/T15683-1995 by Agricultural Products Quality Inspection and Supervision Testing Center of the Ministry of Agriculture (Kunming, Yunnan), China.

DNA extraction

Total DNA was extracted from the leaves of rice plants using the CTAB method described by Murray et al [22].

Detection of the one-step PCR method

The reaction system, amplification program and primers referred to the one-step PCR method by Mao et al [16]. Primer sequences (5´-TCAGGAAGAACAT CTGCAAGG-3´ and 5´-TCAGCTAAACAAACAT AACGAA-3´) were synthesized by Shanghai Bioengineer Ltd Co. Amplification products were subjected to electrophoresis on 3% agarose gel with Bacteriophage φX174-Hinc II digested DNA as a marker.

Detection of the PCR-Acc I method

The reaction system, amplification program and primers followed the method described by Cai et al [15]. Primer sequences (5´-GCTTCACTTCTGTGCCTGTG-3´ and 5´-ATGATTTAACCGAGTTGAA-3´) were synthesized by Shanghai Bioengineer Ltd Co.

After the PCR reaction, enzymolysis was performed as follows: 12 µL of PCR products, 1.5 µL of 10xbuffer, 5 U Acc I and some ddH2O were added into a 0.5 mL centrifuge tube to a final volume of 15 µL, and the reaction mixture was kept in water-bath at 37°C for 1.5 h. Then the reaction products and respective PCR products before enzymolysis (as controls) were subjected to electrophoresis on 3% agarose gel with Bacteriophage φX174-Hinc II digested DNA as a marker and divided into digested DNA bands and undigested DNA bands by Acc I in view of migration rate.

RESULTS

Comparison of the one-step PCR method and the PCR-Acc I method

When DNAs from 220 rice materials were amplified by the one-step PCR method, some varieties presented one amplified band, while the others only exhibited extremely faint amplification bands or none. In this study, the 3’ terminal base of the used upstream primer was designed as just the base G at position +1 of the donor splice site of waxy gene intron 1. If the relevant site on template DNA was base C, the
amplification efficiency would be 100 times higher than that of the relevant base A on template DNA [16, 23], thereby the amplified products showed a clear 237 bp DNA band by electrophoresis on 3% agarose gel, and its genotype was GG (e.g. Lanes 1, 2, 5, 6, 7, 8, 10, 11, 12, 15 in Fig. 1). However, when the relevant site on template DNA was base A, PCR products just showed extremely faint bands or none on 3% agarose gel, and its genotype was TT (e.g. Lanes 3, 4, 9, 13, 14 in Fig. 1).

To confirm the results of the one-step PCR, the DNAs of the tested materials were amplified by the PCR-Acc I method. DNAs of 101 rice varieties selected randomly were firstly amplified by the primers of PCR-Acc I method. All of the rice materials displayed a 460-bp amplified DNA band with the same migration rate (Fig. 2). Afterwards these amplified DNA bands were digested with Acc I. When the first base of the donor splice site of waxy gene intron 1 is base G, the sequence together with its flanking base sequence GTATAC contains the recognition site of restriction endonuclease Acc I, so the 460-bp DNA band will be digested into a 403-bp band and a 57-bp band after treated with Acc I (Lanes 2’ and 4’ in Fig. 2). While the first base of the donor splice site of waxy gene intron 1 is base T, the sequence with its flanking sequence cannot be recognized by Acc I, consequently the 460-bp DNA band can not be digested by Acc I (Lanes 1’, 3’ and 5’ in Fig. 2). By electrophoresis on 3% agarose gel, the undigested 460-bp band and digested 403-bp band could be distinguished easily based on different migration rates (Fig. 2). When the same 460-bp band could be detected after and before Acc I digestion, the base at the position +1 of the donor splice site of waxy gene intron 1 was base T, thereby the rice variety was recorded as TT genotype; while a 403-bp band was found after Acc I digestion, the base at position +1 of the donor splice site should be G, and the rice variety was GG genotype.

By using the PCR-Acc I method and the one-step PCR method for determining the first base of the donor splice site of waxy gene intron 1, the completely same results were obtained in the 101 rice materials selected randomly from the 220 rice varieties. This verified that the one-step PCR method was reliable to detect the first base of the donor splice site of waxy gene intron 1.
Relationship between the first base of the donor splice site of waxy gene intron 1 and amylose content in rice

According to the base G/T at position +1 of the donor splice site of waxy gene intron 1, the 220 Yunnan indigenous rice varieties could be divided into two genotypes: GG and TT. Of the tested materials, 164 rice varieties belonged to GG-genotype, while the other 56 to TT-genotype, accounting for 74.55% and 25.45%, respectively. Moreover, 80.49% of indica rice and 67.01% of japonica rice showed GG-genotype. It was thus clear that regardless of rice types (indica or japonica), GG-genotype of rice occupied a dominant position in Yunnan indigenous rice varieties, especially in indica rice.

The amylose content and other information of rice varieties with different GG/TT genotypes were showed in Table 1 and Fig. 3. The results showed that the rice varieties with GG-genotype had significantly higher mean amylose content (18.95%) than those with TT-genotype (8.40%) in both indica and japonica rice, suggesting that G/T polymorphism was closely related with amylose content. Meanwhile, results of statistical analysis indicated there existed a significant relationship between genotype and amylose content with a correlation coefficient of 0.733 ($P<0.01$). Moreover, amylose contents of TT-genotype rice were all below 15.92%, whereas most of GG-genotype rice had high amylose contents (18.95% on average), though 19 indica rice and 14 japonica rice with GG-genotype (accounting for 20.12% of total GG-genotype) had low amylose content ranging from 3.91% to 15.93%. Furthermore, the mean amylose contents of the entire, indica and japonica groups with GG genotype were 18.95%, 18.91% and 18.97%, respectively, without significant difference; while those with TT genotype were 8.40%, 8.91% and 8.02%, respectively, also without significant difference. This suggested that different genetic backgrounds, i.e. indica or japonica, had no significant effect on amylose content.

**DISCUSSION**

In this study, the reliability of the one-step PCR method for determining the base at position +1 of the donor splice site of waxy gene intron 1 was verified by the PCR-Acc I method by using 101 rice materials randomly selected from the 220 rice varieties. The PCR-Acc I method required restriction endonuclease Acc I and included two steps of PCR and digestion, increasing experimental cost and time. Compared with the PCR-Acc I method, the one-step PCR method only need PCR and electrophoresis, being simple, economical and more applicable to the massive detection in rice breeding. The one-step PCR method determined rice genotype according as the amplified DNA was clear or faint even none, so some rice varieties with GG genotype might not show clear DNA band and were judged as TT-genotype due to poor results of amplification or electrophoresis. For this reason, we must ensure good amplification efficiency and careful electrophoresis, and repeat experiment and detection on rice materials without clear DNA band.

As a product of coordination among coding sequence, expression and regulation of waxy gene, amylose content is not only controlled by the first base of the donor splice site of waxy gene intron 1, but also

### Table 1. Genotype and amylose content of the test materials.

<table>
<thead>
<tr>
<th>Population</th>
<th>Genotype</th>
<th>No. of varieties</th>
<th>Percent (%)</th>
<th>Amylose content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>Entire</td>
<td>GG</td>
<td>164</td>
<td>74.55</td>
<td>3.91-24.88</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>56</td>
<td>25.45</td>
<td>1.84-15.92</td>
</tr>
<tr>
<td>indica</td>
<td>GG</td>
<td>99</td>
<td>80.49</td>
<td>3.91-24.88</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>24</td>
<td>19.51</td>
<td>2.64-15.92</td>
</tr>
<tr>
<td>japonica</td>
<td>GG</td>
<td>65</td>
<td>67.01</td>
<td>5.16-24.3</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>32</td>
<td>32.99</td>
<td>1.84-15.63</td>
</tr>
</tbody>
</table>

![Fig. 3. Frequency of GG/TT genotype associated with amylose content.](image-url)
affected by some minor genes, modulins and regulatory sequences of waxy gene. Therefore, the coefficient of correlation between the first base of the donor splice site of waxy gene intron 1 ($r=0.733$, $P<0.01$) could not explain all variances of amylose content. In our study, amylose contents of TT-genotype rice were all below 15.92%, while those of GG-genotype rice were high mostly. However, 33 rice varieties with GG-genotype (accounting for 20.12% of total GG-genotype) still had low amylose contents ranging from 3.91% to 15.93%, and most of them came from the Dai minority area in the Southwest of Yunnan Province. Of the 33 rice varieties, 23 rice varieties were distributed over the center area of genetic diversity of Yunnan rice classified by Zeng et al [24], which was also the largest center of genetic diversity for Chinese cultivated rice and natural treasure house of good resources in China: 7 ones were in the diffuse area of genetic diversity around the center area; and the other 3 ones were over the wasteland area of genetic diversity. This suggested that low amylose contents of the 33 GG-genotype rice varieties were probably related to ecological diversity of the center area of rice genetic diversity in the Southwest of Yunnan and the bias of local minority towards the rice with glutinous nature.

Our study indicated that there was no significant difference in the mean amylose contents of the same GG or TT genotypes between indica and japonica rice. This is not agreed with the conclusion by Zhang et al [25], in which indica rice with GG-genotype had significantly higher amylose content than japonica rice with the same genotype. It might be attributed to the difference in experimental materials, i.e. the rice materials used in the study by Zhang et al [25] were highly differentiated indica or japonica rice, whereas our materials were from the center of rice genetic diversity, which might not yet completely differentiate into indica or japonica rice. As a result, under two different genetic backgrounds, indica or japonica, there was no significant difference in the amylose contents of rice materials with the same genotype and the two genetic backgrounds had no significant effect on amylose content.

Hirano et al [13] reported that the first base of the donor splice site of waxy gene intron 1 in common wild rice and indica rice was base G, while japonica rice was base T, so he believed waxy gene of japonica rice was differentiated from waxy gene of common wild rice. At the same time, Yamanaka et al [26] found that the first base of waxy gene intron 1 in all japonica rice is base T. However, other studies [15-16, 25, 27] showed that there are not only base G but also base T in indica rice, and that japonica rice have also base G from the results of Zhang et al [25] and He et al [27]. As far as our experiment concerned, we found that regardless of indica or japonica rice, the rice materials with base G at the first base of the donor splice site of waxy gene were predominant in Yunnan indigenous rice varieties. Whether this meant that Yunnan indigenous rice varieties have more primal waxy gene and are not yet thoroughly differentiated into indica or japonica rice, further studies should be made.

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