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Multiplex PCR analysis of *Indica-japonica* differentiation of the chloroplast DNA in weedy rice

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Abstract: The whole chloroplast genomes of the *Indica* cultivar 93-11 and *Japonica* cultivar Peiai64S were analyzed and compared. A multiplex PCR marker of the chloroplast DNA ORF100 and ORF29-TrnC^{GCA} region was optimized and constructed. More than 200 weedy rice, Asian cultivated rice and common wild rice accessions were then tested using the multiplex PCR. The results showed that there were obvious chloroplast DNA *indica-japonica* differentiations amongst the weedy, Asian cultivated and common wild rice accessions. Furthermore, the differentiations in weedy rice were correlated to the collected regions that were in accordance with the south *indica* type and north *japonica* type of cultivated rice in China. It was suggested that the *japonica* line of weedy rice should evolve from degraded cultivated rice or the *japonica* varieties(as the female parent) natural hybridization with other *oryza* materials for there was no wild form in north China.

Key words: weedy rice; chloroplast DNA; *Indica-Japonica* differentiation; multiplex PCR; hybridization
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杂草稻叶绿体籼粳分化的多重 PCR 分析

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摘 要: 通过分析籼稻 93-11 和粳稻培矮 64S 的叶绿体全基因组,优化和构建了籼粳分化的叶绿体分子标记 ORF100 和 ORF29-TrnC^{GCA} 的多重 PCR。应用这个多重 PCR 对 200 余份世界各地杂草稻和其它水稻材料进行分析。结果表明:杂草稻中有明显的叶绿体籼粳分化,表现出明显的地域性,且与传统的中国栽培稻的南籼北粳能较好的对应。推测粳型杂草稻可能是栽培稻突变或粳型水稻(作母本)与其它类型水稻材料杂交而形成的。

关键词: 杂草稻; 叶绿体 DNA; 籼粳分化; 多重 PCR; 杂交

Weedy rice(*Oryza sativa* f. *spontanea*), as a competitive weed with cultivated rice(*Oryza sativa*), is widely distributed in rice-planting filed over the world, particularly in south and southeast Asia, south and north America, northern Australia, and southern Europe, where the direct seeding or no-till technology is usually applied to rice farming(Briana *et al.*, 2010; Tang *et al.*, 2011). The growth of weedy rice in cul-

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tured rice fields could cause significant reduction in rice yield and affect the quality of rice grains because of its great growing ability and persistency in rice fields (Carrie *et al.*, 2010; Zuo *et al.*, 2011). Compared with other weeds, weedy rice is very difficult to control in cultivated rice fields because it shares similar morphological features, physiological traits and herbicide tolerances with the cultivated rice. Furthermore, weedy rice and cultivated rice can hybridize owing to the genetic similarity, and potential environmental consequences of weedy rice from introgression following the commercial release of genetically modified rice (Song *et al.*, 2011; Xia *et al.*, 2011). To date the general strategy to control weedy rice is to practice preventative, cultural, mechanical and chemical means, such as crop rotation and herbicide application. However, the weedy rice has been considered a promising and useful reservoir for genetic variation to rice improvement, because it has many useful genetic characteristics, such as disease resistance, drought and chilling tolerance, competitive ability, etc., and there is no apparent genetic barriers between weedy rice and cultured rice. Moreover, weedy rice is successfully acclimatized to the natural growing conditions (Burgos *et al.*, 2011).

Weedy rice is taxonomically classified and nominated as *Oryza sativa* f. *spontanea* being the same genus and species as Asian cultivated rice by morphological characters and physiological traits. Many studies have demonstrated that high genetic differentiation in rice genomes occurred in Asian cultivated rice and its wild ancestor *Oryza rufipogon*, in which the significant differentiation is *indica*-*japonica*. The *indica*-*japonica* variation occurred both in the nuclear genome and in chloroplast DNA of Asian cultivated rice, presumably in the weedy rice due to the conspecificity of Asian cultivated rice (Xiong *et al.*, 2010; Zuo *et al.*, 2011).

In order to effectively utilize the beneficial genes and facilitate the control strategies, it is of the oretical and practical significance to fully understand and clarify the characteristics of *indica*-*japonica* differentiation in the weedy rice. However, the differentiation research in weedy rice is still limited, especially in the chloroplast DNA. The most common method for identification of *indica* and *japonica* rice varieties is Chen's index that examines six key characters of rice samples. However, those morphological and physiological characters can be influenced greatly by the change of environmental conditions. In addition, it is necessary to measure the concerned characters from mature rice plants when this morphological-based *indica* and *japonica* identification method is used. Therefore, the long time is needed to generate the results, which has considerably limited its wide application for *indica* or *japonica* rice identification (Xiong *et al.*, 2010).

The sequencing of the chloroplast genome of *japonica* rice cv. Peiai64 (PA64S, AY522331.1) and *indica* rice cv. 93-11 (AY522329.1) has been completed in 2004. The completion and global availability of total rice chloroplast genome sequences have made the development of specific molecular markers possible using tools of comparative genomics that are essentially based on the differences of entire genomic sequences between *indica* and *japonica* rice. Based on the insertion and deletion (InDel) fragments obtained in chloroplast DNA sequences between PA64S and 93-11, the ORF100 bands of *japonica* rice lags behind most of *indica* rice because there is a 69 bp deletion at 8 549-8 617 bp in the *indica* while most *japonica* cultivars do not have it. This region has been considered to be an effective marker for *indica*-*japonica* differentiation. Tang *et al.* (2004) found that the *indica* type rice had a 32 bp insertion at 17 709 - 17 740 bp in the ORF29-TrnC^{GCA} spacer but the *japonica* type did not contain such an insertion, leading to a lag of the *indica* bands related to the *japonica* ones. Therefore, ORF29-TrnC^{GCA} can be regarded as another marker to distinguish the *indica* chloroplast genome from that of *japonica* (Ou *et al.*, 2009). Multiplex-polymerase chain reaction (multiplex PCR) is a novel technique in which several pairs of primers are used together in one PCR reaction leading to simultaneous amplification and detection of different regions or sizes of DNA fragments. It will be a very useful and effective protocol for genetic differentiation.

The main objective in this study was: (a) to develop and assess a new multiplex-PCR marker of

above two mentioned markers for reliable and low-cost screening the different rice materials; (b) to investigate and reveal the differentiation in weedy rice populations from different localities with the multiplex-PCR; and (c) to explore the origin of weedy rice by *indica*-*japonica* differentiation.

1 Materials and Methods

1.1 Plant material and DNA extraction

One hundred and fifty-four weedy rice accessions were collected by Shenyang Agricultural University (SYAU) between 2005 and 2007 in rice-planting areas of China in which thirteen regions from Liaoning Province, two from Jilin Province, two from Heilongjiang Province and one from Hubei Province. Moreover, 49 weedy rice accessions from abroad, and Hubei and Jiangsu provinces in China were obtained from Yunnan Agricultural University, China (YNAU) and China National Rice Research Institute (CNRRI). In total, 227 rice accessions, including 203 weedy rice accessions, 8 *indica* rice varieties, 8 *japonica* rice varieties and 8 common wild rice accessions were selected for this study. The 93-11 and PA64S were included as a reference, representing typical *indica* and typical *japonica*. DNA was extracted according to Ou *et al*. (2009).

1.2 Primers design

PCR primers were designed by Primer Premier 5.0 based on the whole chloroplast genome sequence of 93-11 or PA64S. ORF29-TrnC^{GCA} marker primer pair amplification products were designed at 17 620-17 980 bp of PA64S and 17 546-17 938 bp of 93-11 whereas ORF100 primer pair designed at 7 897-8 745 bp of PA64S and 7 903-8 682 bp of 93-11. Detailed information of the primer pairs is given in Table 1.

1.3 Multiplex PCR analysis

The multiplex PCR assays were carried out as the following reagent concentrations: 50 ng genomic template DNA; 2.0 μL 10 × Taq PCR buffer; 2.0 mmol/L of $MgCl_2$; 125 μmol/L of each dNTP; 1.0 U of Taq DNA Polymerase (MBI Fermentas Inc., USA); 0.5 μmol/L of each forward and reverse primer pair (Sangon Company, Shanghai). The final reaction volume was adjusted to 20 μL with the sterilized ultrapure dddH_2O.

表 1 引物的序列和目标片段的预期大小

Table 1 Primer sequence, target fragment and their expected product sizes

Primer	Orientation	Primer sequence (5′-3′)	Target fragment	Size of product (bp)	
				Indica	*Japonica*
P1	Forward	A AGGCTCGGCGATACTG	ORF29-TrnC^{GCA}	393	361
P2	Reverse	GCAGCCCAAGCGAGACT			
P3	Forward	AGCCGAGGTCGTGGTAA	ORF100	780	849
P4	Reverse	AGTCCACTCAGCCATCTCTC			

Amplifications were done using a PTC 225 Peltier Thermal Cycler (MJ Research Inc. USA). The amplification profile was performed with a touchdown PCR program as following, 5 min at 94 ℃; 10 cycles of 45 s at 94 ℃, 45 s at 60 ℃ (minus 0.5 ℃/cycle), 1 min at 72 ℃; 25 cycles of 45 s at 94 ℃, 45 s at 55 ℃, 1 min at 72 ℃; and 10 min at 72 ℃ for final extension, 4 ℃ for holding. The PCR products were resolved using 1.5% agarose gel electrophoresis.

1.4 Data analysis

Extensive multiplex PCR analyses were conducted to investigate variation patterns of the rice accessions. Then the *indica*-*japonica* differentiation of the chloroplast DNA in rice would be analyzed and determined based on the multiplex-PCR amplified profile. The electrophoretic banding patterns of the sequenced *indica* rice variety (93-11) and *japonica* variety rice (PA64S) were used as a reference for determining the *indica* or *japonica* genotype, respectively. Consequently, the banding pattern was scored as *indica*-genotype (I) if it was identical to that of 93-11, or as *japonica*-genotype (J) if it was identical to that of PA64S, or as *indica*-*ja*-

ponica-genotype(IJ) if the banding pattern was identical to that of 93-11 and PA64S.

2 Results and Analysis

2.1 Analysis of the ORF29-TrnC^{GCA} and ORF100 marker

To validate the effectiveness of chloroplast ORF29-TrnC^{GCA} and ORF100 InDel molecular marker for the identification of *indica* and *japonica* rice varieties, four traditional *indica* rice(93-11, IR24, Qishanzhan and Ezao18) and four traditional *japonica* rice(PA64S, Nipponbare, SN265 and SN6014) samples from different localities, were analyzed to examine the polymorphisms.

The ORF100 PCR products amplified by the P3+P4 primer pair revealed that all the *indica* rice is consistent with the reference *indica* rice 93-11. In contrast, the ORF100 bands of traditional *japonica* rice lag behind 93-11 and are consistent with the reference *japonica* rice PA64S. Additionally, the results from the ORF29-TrnC^{GCA} analysis using P1+P2 primer pair are also compatible with the ORF100, and demonstrate that all the bands of *indica* rice are the same as the 93-11 but *japonica* rice being the same as PA64S. Results from identification of the two primer pairs suggested that they are identical. These results also confirmed the effectiveness of the two InDel molecular markers, which could be used to accurately detect and classify the *indica-japonica* differentiation in the chloroplast DNA of cultivated rice. The partial electrophoresis results were showed in Figure1.

2.2 Multiplex PCR development

The ORF29-TrnC^{GCA} PCR reaction would yield two fragments with 393/361 bp in length for all the rice accessions whereas ORF100 with 849/760 bp. They showed that there was 399-488 bp difference between the two markers. In this study, an attempt was made to develop a multiplex marker system for these two markers to distinguish the *indica-japonica* differentiation in the chloroplast DNA of rice.

Multiplex PCR of the ORF29-TrnC^{GCA} and ORF100 assay was performed and validated using the same varieties as the single PCR. As shown in Fig. 1, the result from the multiplex PCR analysis revealed is absolutely identical to that from the two single PCR assays respectively. The large band of the ORF100 is combined with the small band of the ORF29-TrnC^{GCA} in *japonica* rice being contrary to that in *indica* rice. This would enlarge the relative difference between the two bands of the single PCR and avoid the negative results for little difference with 32 bp or 69 bp. The result demonstrated that the multiplex PCR marker would provide an effective and accurate method for identifying a large number of rice varieties for their *indica* and *japonica* characteristics.

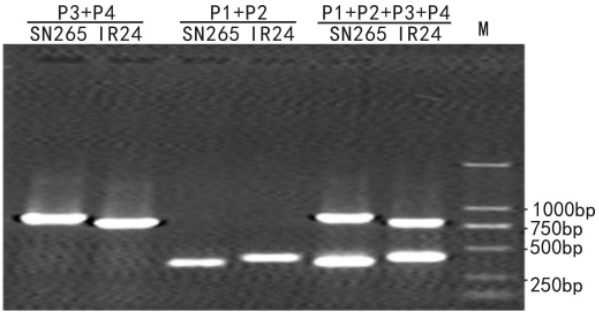

图 1　叶绿体标记的单重和多重 PCR 电泳图

Fig. 1　Electrophoresis of single and multiplex PCR products amplified with the chloroplast markers

2.3 *Indica-japonica* differentiation of the rice accessions

To evaluate the *indica-japonica* differentiation among the rice accessions, extensive multiplex-PCR molecular analyses were conducted to investigate variation patterns of the 227 rice accessions from different regions. The results showed that the *indica-japonica* differentiation was detected in 16 cultivated rice materials and identical to the known type previously. Similarly, out of 8 common wild rice accessions, 4 were *indica* types, and the other 4 were *japonica* types. Results from this study showed that the cultivated and wild *Oryza* rice species or populations had considerable *indica-japonica* differentiation in the chloroplast DNA.

Additionally, among the 203 accessions of the examined weedy rice, most of them, 192 accessions from north China and five foreign countries exhibited typical *japonica* type (Fig. 2) . On the contrary, the remaining 11 from south China(Hubei and Jiangsu province) ac-

cessions showed typical *indica* type. The data from the field experiment also showed that weedy rice from north China had a similar agronomic performance of the typical cultivated *japonica* rice. The data from the field experiment also showed that the weedy rice from North China had a similar agronomic performance of the typical cultivated *japonica* rice (data not shown). These results suggest that the chloroplast DNA of weedy rice should come from *indica* or *japonica* type rice respectively. It is also indicated that the differentiation of the weedy rice is correlated to the collected regions, which are in accordance with the south *indica* north *japonica* cultivated rice in China. It is well recognized that *indica* rice is generally grown in south China, whereas *japonica* rice is exclusively grown in north China indicating that weedy rice in China should be polyphyletic evolution.

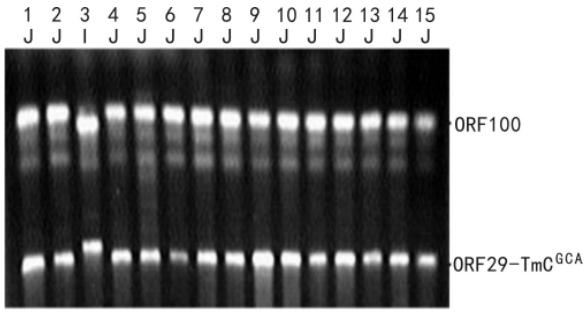

图 2 部分杂草稻材料的多重 PCR 扩增效果图
Fig. 2 PCR patterns amplified by the multiplex PCR in partial weedy rice

3 Conclusion and Discussion

As shown above, this is the first report on a fast and reliable multiplex chloroplast DNA PCR marker based on the insertion and deletion (InDel) fragments obtained in DNA sequences between the entire chloroplast genomes of the typical *indica* rice variety 93-11 and *japonica* variety rice PA64S for the rice *indica-japonica* differentiation analysis. It can provide an effective and accurate method to identify a large number of rice varieties for their *indica* and *japonica* characteristics in a short time and any laboratory can do the identification with PCR facilities and electrophoresis. The clear understanding *indica-japonica* differentiation of the chloroplast DNA in weedy rice is of significant benefit to rice breeders.

Despite of the fact that weedy rice is the sample species complex as the Asian, its evolutionary origin is still unclear. Asian cultivated forms mainly show two types, referred as subspecies *indica* and *japonica*. In eco-geographical terms, *indica* rice varieties are known to be adapted to the tropical and subtropical environments at low latitudes or altitudes with warm climate conditions, but *japonica* rice varieties are adapted to the temperate environment at high latitudes or altitudes with relatively cool conditions. In addition, *Indica* strains are predominantly distributed from south to southeast Asia, whereas *japonica* strains are cultivated in insular regions, mountain areas, and North countries (Xiong *et al*., 2010). It is concluded that the weedy rice differentiation is associated with the selected regions. Data from the present experiment suggest that the *japonica* line of weedy rice may be evolved from the degraded cultivated rice or the *japonica* varieties (as the female parent) natural hybridization with other *Oryza* materials for there is no wild form in north China and the weedy rice in China may have different origins. However, the information obtained from this study is an initial step toward understanding the origin of weedy rice, and it needs further research for elucidating their origins. In summary, the weedy rice of the different regions or biotypes may have originated in different ways (Xia *et al*., 2011; Zhang *et al*., 2012).

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显著影响 PEG 6000 胁迫下水稻幼根的长度(图 1: B),表明 PEG 6000 胁迫下水稻幼根的生长和过氧化氢水平没有明确关联。

2.3 PEG 6000 胁迫下 SHAM 对水稻幼根的影响

用 1 mmol·L^{-1} SHAM(使用浓度参照 Bartoli *et al.*,2005),预处理水稻幼根并将幼根置于 PEG 6000 下培养后发现(方法同 DMTU),SHAM 的处理使得 PEG 6000 胁迫下水稻幼根过氧化氢含量进一步上升(图 2: A)。本研究也发现,SHAM 进一步导致了 PEG 6000 胁迫下水稻幼根细胞死亡水平的上升和相对含水量的下降(图 2: C,D),表明该抑制剂会降低植物对 PEG 6000 所引起的渗透胁迫的耐受。本研究也发现,SHAM 的处理也导致了 PEG 6000 胁迫下水稻幼根生长的进一步降低(图 2: B)。

3 讨论

SHAM 是交替氧化酶的抑制剂,交替氧化酶的存在能通过降低线粒体电子传递链的过度还原而限制线粒体中过氧化氢的生成(Viacheslav *et al.*,2011; Millenaar *et al.*,2003);尤其作为非光合组织,线粒体在理论上是根在渗透胁迫下产生 H_2O_2 的主要位点(De Carvalho,2008)。因而,本研究认为,SHAM 可能是通过抑制交替氧化酶而进一步刺激了渗透胁迫下植物 H_2O_2 的生成。介于以上关于 PEG 6000 胁迫下水稻幼根相对含水量和细胞死亡与过氧化氢关系的观察,推测 SHAM 对水稻幼根渗透胁迫耐受性的影响可能也和其刺激了植物过氧化氢的生成有关。实验结果表明在 PEG 介导的渗透胁迫下,交替氧化酶也和植物幼根的生长存在着一定的联系,但其内在机理尚需要进一步研究。

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