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ARTICLE

A multiplex PCR system for detection of wide compatibility allele *S5-n* and erect panicle allele *dep1* in rice

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Abstract: *Wide compatibility and erect panicles are important traits for heterosis and ideal plant type breeding in rice and accordingly are targets for rice yield improvement. In this study, a multiplex PCR system based on functional markers was developed to simultaneously identify genotypes of the wide compatibility allele S5-n and the erect panicle allele dep1. Forty-nine rice varieties in the Huang-Huai-Hai region of China were identified using this system. Thirty-three varieties contained the erect panicle allele dep1 and two varieties contained the wide compatibility allele S5-n. The frequency of dep1 was obviously higher than that of S5-n. The multiplex PCR method was also tested using an F_2 segregating population and was found to be simple, efficient, and reliable. It will be useful in marker-assisted selection based on the wide compatibility allele S5-n and erect panicle allele dep1 to breed super rice varieties using indica and japonica heterosis.*

Key words*: Functional marker, S5-n, DEP1, multiplex PCR.*

INTRODUCTION

Rice is one of the most important food crops worldwide. The continuous improvement of rice yield is an important way to ensure food security (Liang et al. 2014). According to Chen et al. (2001), rice yield improvement in the future will depend on super high-yield rice breeding that combines ideal plant type and heterosis. *Indica* and *japonica*, two subspecies of Asian cultivated rice (*Oryza sativa* L.), show strong F_1 heterosis, and accordingly have the potential to produce super hybrid rice plants (Konishi et al. 2006, Qian et al. 2016). However, owing to the high genetic distance between *indica* and *japonica*, the F_1 hybrids generally have low fertility, which is a major obstacle in the utilization of inter-subspecific heterosis. Accordingly, overcoming the problem of hybrid sterility between *indica* and *japonica* to improve yield is an important topic in rice breeding research.

Ikehashi and Araki (1986) proposed the wide compatibility theory and mapped the *S5* locus for *indica-japonica* hybrid sterility to chromosome 6. There are three alleles at the *S5* locus, i.e., the *indica* allele *S5-i*, the *japonica* allele *S5-j*, and the neutral allele *S5-n* (referred to as the wide-compatibility allele). Plants with the S5-n/S5-j (or S5-i) genotype are fully fertile, while plants with the S5-i/S5-j genotype have low fertility. Chen et al. (2008) cloned the *S5* gene from the wide-compatibility variety 02428 using a map-based cloning

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approach and characterized its function. The *S5* of *indica* and *japonica* differ by two nucleotides, resulting in an amino acid difference and thus leading to hybrid F_1 sterility. A 136-bp deletion in *S5* including the translation start site in the wide-compatibility variety results in the functional loss of aspartic protease, leading to a lack of a reproductive barrier between *indica* and *japonica*. The successful cloning of the wide compatibility allele *S5-n* facilitates gene exchange and the utilization of *indica* and *japonica* heterosis.

An erect panicle is an ideal plant type trait in high-yield rice (Qian et al. 2016, Zhao et al. 2016). Erect panicle varieties with short, dense panicles, straight leaves, and a small leaf angle could improve the rice population structure and thus increase the efficiency of photosynthesis and the accumulation of photosynthetic products (Xu et al. 1995, Cheng et al. 2011). Huang et al. (2009) cloned the *DEP1* from the super rice variety Shennong 265 using a map-based cloning method. The *DEP1*, including 5 exons and 4 introns, encodes amino acids that are functionally similar to the phospholipid diethanolamide binding protein. The erect panicle allele *dep1* has a 625-bp deletion in the fifth exon of *DEP1* resulting in the premature termination of transcription. The mutated *dep1* allele could enhance rice yield by improving panicle density, branch number, grain number per panicle, and nitrogen uptake and metabolism.

DNA marker-assisted breeding combines molecular markers and crop breeding. The technique has many advantages, such as its insensitivity to environmental conditions, high reliability, and time-efficiency (Dudley et al. 1993, Ribaut et al. 1998, Schuster 2011, Xu 2013). A functional marker is designed according to polymorphic sites within gene sequences affecting phenotypic variation (Andersen and Lübberstedt et al. 2003). Compared with the traditional marker-assisted selection, functional markers have apparent advantages for the detection of the target trait allele in both artificial and natural selection populations (Andersen and Lübberstedt 2003, Varshney et al. 2005). The multiplex PCR functional marker system has various benefits, including its favorable accuracy, repeatability, and efficiency (Varshney et al. 2005, Ramkumar et al. 2010, Salgotra et al. 2011). In this study, a functional marker-based multiplex PCR assay for the simultaneous detection of *S5-n* and *dep1* was developed and was used to detect 49 rice varieties in the Huang-Huai-Hai region of China and a F_2 segregating population derived from a cross between *indica* and *japonica*. The results indicated that the system could be applied to the rapid detection of *S5-n* and *dep1* in rice germplasm resources and to molecular marker-assisted selection in *indica-japonica* hybrid breeding.

MATERIAL AND METHODS

Plant material

Forty-nine *japonica* varieties in the Huang-Huai-Hai region of China and one F_2 population derived from a cross between *japonica* Huaidao 6 and *indica* 9311 were used to evaluate the multiplex PCR system. Information about the 49 varieties is provided in Online Resource 1 and additional information can be found at the China Rice Data Center (http://www.ricedata.cn/index.htm). The wide-compatibility variety 02428 was used as the positive control for *S5-n* and Nipponbare, an incompatible *japonica* variety with the *S5-i* genotype, was used as the negative control for *S5-n*. Wuyunjing 8, an erect and dense panicle variety, was used as the positive control for *dep1* and Nipponbare, a non-erect and non-dense panicle variety, was used as the negative control. From fresh leaves of 3-week-old seedlings, DNA was extracted using the modified CTAB method (Rogers et al. 1989).

Development of functional markers

One deletion of 136 bp near the upstream and downstream regions of the translation initiation site (ATG) differentiates the wide compatibility allele *S5-n* and the incompatibility alleles *S5-j* or *S5-i* and leads to a loss of function of the *S5* (*Os06g0213100*). The primer pair S5-1 for *S5* was designed according to the flanking sequence of the 136-bp deletion (Figure 1). For *DEP1* (*Os09g0441900*), a 625-bp deletion in the fifth exon distinguishes the erect panicle variety from the non-erect panicle variety. The primer pair DEP1-1 for *DEP1* was designed according to the sequences upstream and downstream of the fifth exon (Figure 2). Primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Multiplex PCR amplification and detection

The multiple PCR reaction system for *S5-n* and *dep1* used a reaction volume of 20 µL, containing 10 µL of 2×Es Taq Master Mix (including 0.1 U μL^{-1} Es Taq DNA polymerase, 2×Es PCR Buffer, 3 mM $MgCl_2$, and 0.4 mM dNTP mix, provided

Figure 1. Location of the primers and deletion sequence in *S5-n*. The dashed line indicates the deletion in *S5-n*; arrows indicate the primers; red letters indicate the translation start site; *S5* indicates *Os06g0213100*.

by Beijing ComWin Biotech Co., Ltd.), 2 µL (40-60 ng μL^{-1}) of template DNA, 1 µL (0.4 µmo1 L^{-1}) of forward primer for each gene, 1 µL (0.4 µmo1 L^{-1}) of reverse primer for each gene, and 4 µL of ddH_2O. The PCR was performed using a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). The PCR conditions were as follows: 95 °C for 5 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min, 30 s; 72 °C for 8 min. PCR products were then separated on 1% agarose gel containing DNA green in 1× TAE buffer at 120 V for 30 min. The resulting bands were visualized under ultraviolet light and recorded using Gel Doc™ EZ imager (BIO-RAD).

RESULTS AND DISCUSSION

Based on the comprehensive consideration of factors that influence PCR for the two genes, e.g., PCR product length, annealing temperature, and PCR byproducts, sequence polymorphisms were used to design functional markers for *S5-n* and *dep1*. The primer pair S5-1 was designed according to the 136-bp deletion in *S5* of the wide-compatibility variety compared to the incompatible variety. The primers amplified a 321-bp fragment in the wide-compatibility variety and a 457-bp fragment in the incompatible variety (Figure 1). The primer pair DEP1-1 was designed according to the 625-bp deletion in *DEP1* in the erect panicle variety compared to the non-erect panicle variety. The primers amplified a 1235-

Figure 2. Location of the primers and deletion sequence in *DEP1*. The dashed line indicates the deletion in *DEP1*; dots indicate common bases between the two varieties, which were omitted; arrows indicate the primers; *DEP1* indicates *Os09g0441900*.

bp fragment in the erect panicle variety and a 1860-bp fragment in the non-erect panicle variety (Figure 2). Finally, two pairs of primers, S5-1 and DEP1-1, yielding clear and differential PCR products with similar annealing temperatures were obtained.

By the continuous optimization of the annealing temperature, number of reaction cycles, and DNA template concentration, the optimum PCR reaction conditions were obtained, i.e., an annealing temperature of 56 °C, 35 cycles, and a template concentration of 4-6 ng μL^{-1}. Using agarose gel electrophoresis, four amplification products of 321, 457, 1235, and 1860 bp for *S5-n* in the wide-compatibility variety, *S5-(i or j)* in the incompatible variety, *dep1* in the erect panicle variety, and *DEP1* in the non-erect panicle variety were clearly observed.

The multiple PCR system was used to detect the distribution of *S5-n* and *dep1* genotypes in 49 rice varieties recently grown in the Huang-Huai-Hai region of China (Figure 3). Huaidao 6 and Handao 277 had the same 321-bp band as that observed for the wide compatibility control variety 02428, indicating that 4.08% were wide-compatibility varieties. With respect to *dep1*, 33 of 49 varieties had the same 1235-bp band as the erect panicle control variety Wuyunjing 8, indicating that 67.3% were erect panicle varieties. Only Huaidao 6 had both wide compatibility allele *S5-n* and erect panicle allele *dep1* (Table 1).

The multiplex PCR system was applied to an F_2 population derived from Huaidao 6 and 9311. Twenty individual plants were randomly selected from the F_2 population for detection (Figure 4). Two individuals (2 and 6) were homozygous for *S5-n* and *dep1*. Three individuals (10, 12, and 20) were homozygous for *S5-i* and *DEP1*. Six individuals (1, 5, 8, 15, 16, and 19) were heterozygous for *S5* and *DEP1*. Other individuals had one homozygous gene and one heterozygous gene. The results indicated that the multiplex PCR system can be used to detect different *S5* and *dep1* genotypes in F_2 segregating populations.

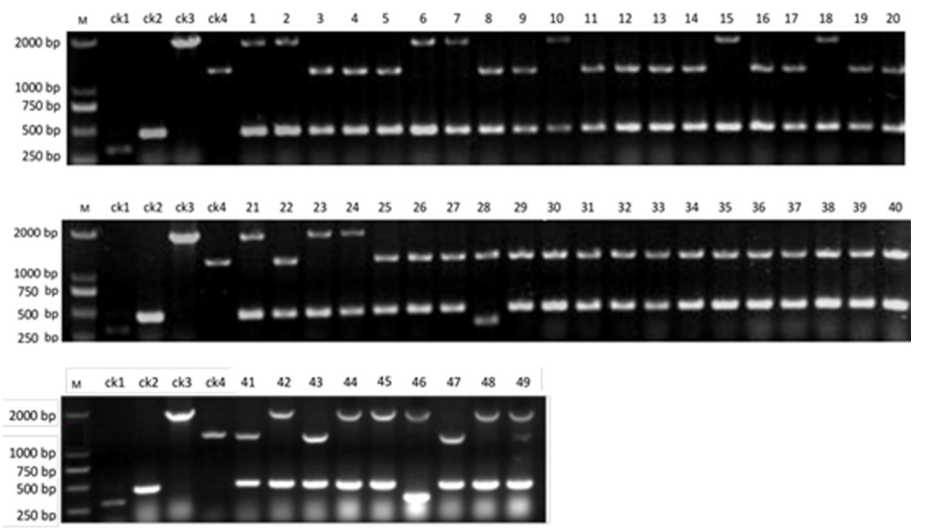

Figure 3. Pattern of PCR amplification obtained using the multiplex PCR system for 49 rice varieties. M indicates the DNA marker; ck1 indicates the positive control for *S5-n*; ck2 indicates the incompatible *japonica* variety with the *S5-i* allele as the negative control for *S5-n*; ck3 indicates the non-erect and non-dense panicle variety with the *DEP1* allele as the negative control for *dep1*; ck4 indicates the erect and dense panicle variety with the *dep1* allele as the positive control. Samples 1-49 are the 49 rice varieties described in Supplemental Table S1; *S5* indicates *Os06g0213100*; *DEP1* indicates *Os09g0441900*.

Table 1. Name, sequences, and expected fragment size for functional markers

Gene	**Primer**	**Sequence** (5'-3')	**Fragment size** (bp)
S5 (*Os06g0213100*)	S5-1 F	GGCAATATGATATGACCAGCAC	457/321
	S5-1 R	ATGTAGCGAAGACAAAGGGAGT	
DEP1 (*Os09g0441900*)	DEP1-1 F	TTTCGGTGGATCGGGTAT	1860/1235
	DEP1-1 R	CGTGACACCGCTAATCGT	

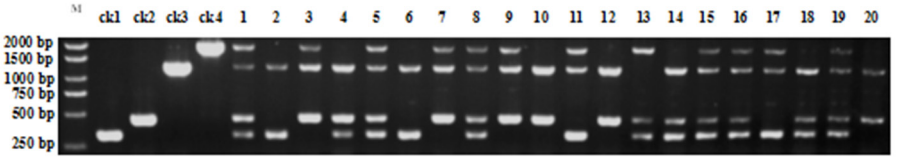

Figure 4. Pattern of PCR amplification obtained using the multiplex PCR system for an F_2 population derived from a cross between *japonica* Huaidao 6 and *indica* 9311. M indicates the DNA marker; ck1 indicate the positive control for *S5-n*; ck2 indicate the incompatible *japonica* variety with the *S5-i* allele as the negative control for *S5-n*; ck3 indicate the erect and dense panicle variety with the *dep1* allele as the positive control; ck4 indicate the non-erect and non-dense panicle variety with *DEP1* allele as the negative control for *dep1*. Samples 1–20 are the 20 individual plants that were randomly selected from the F_2 population; *S5* indicates *Os06g0213100*; *DEP1* indicates *Os09g0441900*.

Using molecular marker technology, a large number of functional alleles controlling important agronomic traits in rice have been successfully located or cloned, providing an impetus for super rice breeding. The combination of heterosis and ideal plant type is an important breakthrough in super rice breeding. The wide compatibility allele *S5-n* and erect panicle allele *dep1* are important for heterosis and ideal plant type breeding in rice. Conventional breeding methods to select plants based on *S5-n* and *dep1* require tedious, time-consuming, and inefficient work related to phenotyping and determining offspring fertility. Thus, a simple and efficient detection system for *S5-n* and *dep1* is required for super rice breeding. Multiplex PCR technology can be used to construct an effective diagnostic system (Chen et al. 2015). Recently, multiplex PCR was successful applied to detect several genes in rice, such as *xa13*, *Xa21*, and *fgr* (Salgotra et al. 2011), *fgr* and *Wx* (Cheng et al. 2015), and *Xa4*, *xa5*, *Xa7*, *xa13*, and *Xa21* (Yap et al. 2016). Here, the multiplex PCR system based on the functional markers was a reliable and sensitive method for the simultaneous detection of *S5-n* and *dep1*. The system could precisely detect plants with *S5-n* and *dep1* by one-time conventional PCR amplification and agarose gel electrophoresis. All results demonstrated that the method is rapid, has high repeatability, and is efficient for identifying *S5-n* and *dep1* in germplasm and for gene-based selection in rice breeding programs, irrespective of plant growth stage, tissue type, and variety.

Compared with conventional PCR, the establishment of a multiplex PCR system is more challenging; it requires a comprehensive analysis and repeated optimization (Chen et al. 2015). Based on the characteristics of the target gene and PCR product, it is essential to design appropriate primer combinations and establish a suitable PCR reaction system. In our opinion, primer design is the most critical step. The annealing temperature should be the same or similar and the length differences between the PCR product of multiplex primers should be distinct. Other factors, such as PCR cycles, template DNA concentration, Taq DNA polymerase, and dNTP concentrations should also be repeatedly tested and modified. In this study, after the repeated optimization procedure, four specific bands of 321, 457, 1235, and 1860 bp for *S5-n* in the wide-compatibility variety, *S5-(i or j)* in the incompatible variety, *dep1* in the erect panicle variety, and

Table 2. The detection results for the multiplex PCR system for 49 rice varieties. "+" indicates the positive detection of the wide-compatibility allele *S5-n* or the erect panicle allele *dep1*, "-" indicates otherwise. *S5* indicates *Os06g0213100*; *DEP1* indicates *Os09g0441900*

Cultivars	Gene		Cultivars	Gene		Cultivars	Gene		Cultivars	Gene	
	S5	*DEP1*		*S5*	*DEP1*		*S5*	*DEP1*		*S5*	*DEP1*
Yunongjing 6	-	-	Zhengdao 11	-	+	Nanjing 5055	-	+	Wuyunuo 6	-	+
Fangxin 1	-	-	Baixiangjing	-	-	Huaidao 6	+	+	Wuyunuo 16	-	+
Xinfeng 2	-	+	Fangxin 4	-	+	Nanjing 42	-	+	Zhenghan 9	-	-
Yujing 5	-	+	Zhengjing 107	-	+	Zhendao 99	-	+	Luodao 998	-	+
Xindao 19	-	+	Zhengdao 5	-	-	Yanfeng 2	-	+	Zhenghan 2	-	-
Zhengdao 4	-	-	Zhengdao 8	-	+	Yandao 11	-	+	Zhenghan 6	-	-
Zhengdao 1	-	-	Zhendao 88	-	+	Wuyunjing 21	-	+	Handao 277	+	-
Zhengdao 19	-	+	Yujing 6	-	-	Jindao 372	-	+	Handao 297	-	+
Yuandao 108	-	+	Xindao 18	-	+	Nanjing 9108	-	+	Handao 502	-	-
Zhudan 5	-	-	Shuijing 3	-	-	Lianjing 11	-	+	Xinhan 2	-	-
Guangcan 1	-	+	Huangjinqing	-	-	Wuyunjing 23	-	+			
Xinfeng 5	-	+	Huaidao 9	-	+	Huaidao 8	-	+			
Hongguangjing 1	-	+	Suxiu 10	-	+	Wuyunjing 2845	-	+			

Online Table 1. Name, approval date, and pedigree of 49 *japonica* rice varieties

No	Variety	Approval	Pedigree
1	Yunongjing 6	2010	Zhongguo 91 / 8902
2	Fangxin 1	2006	Zheng 754 / Yujing 4
3	Xinfeng 2	2007	Yujing 6 / Xinfeng 9402
4	Yujing 5	1994	Taizhong 31 / Zhengjing 12
5	Xindao 19	2009	NJ979 / Yujing 6
6	Zhengdao 4	1992	Unknown
7	Zhengdao 1	1992	Unknown
8	Zhengdao 19	2008	Yunongjing 6 /Z heng 90-36
9	Yuandao 108	2009	(Zhendao 88 / Fu 2115) / (Yujing 7 / Yuan 94134)
10	Zhudan 5	1963	Zhuganqing Family Breeding
11	Guangcan 1	2010	(Yujing 6 // Yujing 7 / Huangjinqing) / Dongjun 5
12	Xinfeng 5	2010	Yujing 6 / Qiu Feng
13	Hongguangjing 1	2005	Yujing7 / Huangjinqing
14	Zhengdao 11	2004	Unknown
15	Baixiangjing	2001	Unknown
16	Fangxin 4	2008	Wuyujing 3 / Baixiangjing
17	Zhengjing 107	1985	Zhengjing 12 Family Breeding
18	Zhengdao 5	1995	Yujing 1 / Shuiyuan 287
19	Zhengdao 18	2008	Zhengdao 2 / Zhengdao 5
20	Zhendao 88	1997	Yuezhiguang / 2507-4
21	Yujing 6	1995	85-12 / Zhengjing 81754
22	Xindao 18	2007	Yan jing 334-6 // Jinxing 1 / Yujing 6
23	Shuijing 3	2002	Zhengdao5 / Huangjinqing
24	Huangjinqing	1993	Nipponbare / Xifeng
25	Huaidao 9	2006	Huai 9712 Family Breeding
26	Suxiu 10	2010	Xiushui 09 / (Bing 00-502 / Xiushui 09)
27	Nanjing 5055	2008	Wujing 13 / Guandong 194
28	Huaidao 6	2000	Wuyujing3 / (Zhongguo 91 / Yanjing 2)
29	Nanjing 42	2006	910 / 178 // Yanjing3 / Yan807 /// Yan265-7
30	Zhendao 99	2001	Zhendao 88 / Wuyujing 3
31	Yanfeng 2	Unknown	Unknown
32	Yandao 11	2012	Zhendao 88 / Yandao 99-1
33	Wuyunjing 21	2007	Yun 9707 / Yun 9726
34	Jindao 372	2014	Zhendao 88 / Jindao 1007
35	Nanjing 9108	2013	Wuxiangjing 14 / Guandong 194
36	Lianjing 11	2012	Zhongye / HA20
37	Wuyunjing 23	Unknown	Unknown
38	Huaidao 8	2004	Wuyunjing 3 Family Breeding
39	Wuyunjing 2845	Unknown	Unknown
40	Wuyunuo 6	Unknown	Unknown
41	Wuyunuo 16	2004	642-1-7 / Jling 58
42	Zhenghan 9	2008	IRAT109 / Yuefu
43	Luodao 998	2006	Handao 277 Family Breeding
44	Zhenghan 2	2003	Zhengdao 90-18 / Lushi
45	Zhenghan 6	2005	Zhengzhouzaojing / Zhengdao 92-44
46	Handao 277	2003	Qiuguang / Banli 1
47	Handao 297	2003	Mujiao78-595 / Khaomon
48	Handao 502	2003	Qiuguang / Hongkelaoshuya
49	Xinhan 2	2011	IAPAR9 Family Breeding

DEP1 in the non-erect panicle variety were clearly observed. The results demonstrated the feasibility of our method for multiplex PCR design.

In this study, the erect panicle allele *dep1* was substantially more frequent than the wide-compatibility allele *S5-n* in 49 varieties from the Huang-Huai-Hai region of China. Thirty-three varieties had the erect panicle allele *dep1*, two varieties had the wide-compatibility allele *S5-n*, and only one variety had both alleles. These results indicated that *DEP1* is widely used in the Huang-Huai-Hai region of China and most rice varieties have erect panicles, as observed in the field. In contrast, wide-compatibility varieties were very rare and *S5-n* is seldom used in rice breeding in the Huang-Huai-Hai region of China, limiting the utilization of *japonica* and *indica* heterosis. *S5* is a key gene involved in *indica-japonica* hybrid sterility. Wide-compatibility varieties with *S5-n* are able to overcome reproductive barriers. Thus, they produce highly fertile hybrids when crossed with *indica* or *japonica* varieties, which promotes gene flow and heterosis. The *DEP1* gene is pleiotropic; it is involved in erect panicles, number of grains per panicle, and nitrogen uptake and metabolism (Huang et al. 2009), and could control the efficiency of photosynthesis and nitrogen-use efficiency (Sun et al. 2014). The *dep1* alleles have effects on vascular bundle- and panicle-related traits of rice in both *indica* and *japonica* genetic backgrounds (Xu et al. 2015). According to Li et al. (2016), a mutation of *DEP1* could increase panicle density in *japonica* Zhonghua 11, using the CRISPR/Cas9 system. An elite *indica* restorer line with dense and erect panicles was selected by marker-assisted selection of dep1 alleles from backcross populations (Cheng et al. 2011). Therefore, using *S5-n* and *DEP1* to breed a super rice variety based on heterosis and ideal plant type is highly significant. Based on the distributions of *S5-n* and *dep1* in the Huang-Huai-Hai region of China, the utilization of *S5-n* needs to increase to develop more wide-compatibility lines. Selection based on *S5-n* and *dep1* to breed elite rice lines with wide compatibility and erect panicle traits may also be useful for super rice breeding by the combined application of heterosis and ideotype.

CONCLUSIONS

A multiplex PCR system for simultaneously detecting genotypes of the wide compatibility allele *S5-n* and the erect panicle allele *dep1* was developed. This method was found to be simple, efficient, and reliable for 49 rice varieties in the Huang-Huai-Hai region of China and one F_2 segregating population. The *dep1* allele has already been widely exploited in *japonica* rice breeding, unlike the rather scarce allele *S5-n,* which needs to be included more frequently in breeding, to obtain more incompatible varieties in the Huang-Huai-Hai region of China.

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