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Different maternal origins of Japanese lowland and upland rice populations

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Abstract Plastid subtype ID (PS-ID) sequences were determined from sequence data based on CA repeats between genes *rpl16* and *rpl14* in Japanese lowland and upland cultivars. The PS-ID sequences of Japanese rice cultivars showed that there are different maternal origins between lowland and upland cultivars. One subtype, 6C7A, of PS-ID sequences was predominant in all but one Japanese lowland cultivar and carried a combination of the *indica*-specific subtype 8C8A and *japonica*-specific nuclear markers for the isozyme genotype. It is probably a nuclear-cytoplasmic recombinant resulting from natural out-crossing and succeeding self-pollination. The origin of the plastid was re-confirmed by the existence of an *indica*-specific deletion in the plastid genome. In contrast, the Japanese upland cultivars showed two subtypes, 7C6A and 6C7A, of PS-ID sequences. An upland-specific isozyme allele as a nuclear marker was equally predominant in cultivars carrying each subtype. The existence of these particular upland-specific nuclear and cytoplasmic genotypes suggests that the origin of Japanese upland cultivars is different from that of Japanese lowland cultivars. Cultivars carrying the upland-specific nuclear genotype are common in Southeast Asia, but the combination of the upland-specific nuclear and cytoplasmic genotypes which is the same as the Japanese upland predominant type was found in cultivars only in Taiwan and Indonesia. Japanese upland cultivars are closely related to those cultivars.

Keywords Nuclear genotype · Non-coding cp DNA · Upland rice · *Oryza sativa* L

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Introduction

Rice plants (*Oryza sativa* L.) reveal wide adaptability. The cultivated area ranges from the tropical region to the sub-arctic region. In Japan, rice plants are cultivated in paddy fields and also in upland fields. We previously reported that the group of upland cultivars showed more polymorphism than the lowland population for isozyme genotypes (Ishikawa et al. 1992). Further, upland cultivars are found to carry the upland-specific allele for phosphogluconate dehydrogenase (PGD), which has not been found in lowland cultivars, and no other alleles showed such a difference (Ishikawa et al. 1991). Morphological and physiological traits also revealed such differences (reviewed in Tsunoda 1997). Thus, Japanese lowland and upland cultivars are categorized into two populations in this report. The upland-specific allele is also found to be typical in tropical *japonica* cultivars in Southeast Asia, which belong to the alternative subgroup of the *japonica* type considered by Oka (1953,1958). However, most Japanese upland cultivars revealed morphological and physiological traits, as well as molecular markers intermediate between those of tropical and temperate *japonica* types (Ishikawa et al. 1997). A small part of the upland cultivars revealed temperate *japonica*-type characteristics and the rest revealed tropical *japonica*-type characteristics. This suggests that the Japanese upland rice population originated from heterozygotes generated from out-crosses between tropical and temperate *japonica* cultivars. We do not have any information on where and when such outcrosses happened, and which cultivars participated in the crosses. Furthermore,

we have no information of cytoplasmic differences between tropical and temperate *japonica* cultivars.

The large amount of sequence data enable us to compare plastid sequences among plant species. Linker sequences between rice plastid genes *rpl16* and *rpl14* are known to be moderately variable in comparison with stable plastid sequences in higher plants, but still could be amplified by using a single pair of primers (Nakamura et al. 1997). In this report, plastid subtype ID (PS-ID) sequences based on the polymorphic DNA sequences of the linker sequences (Nakamura et al. 1997) were used to characterise molecular differences between Japanese lowland and upland rice populations, and also between tropical and temperate *japonica* cultivars at the cytoplasmic level. These PS-ID sequences provided a new approach to identify the rice genetic material of the *japonica* cultivars. In addition to the plastid sequences, the nuclear genotype data was combined with the data of the cytoplasmic genotypes. The combined data tell us how particular populations were formed with related rice groups. Within species like *Oryza sativa* L., there are several sequence variants. Three types, 7C7A, 8C8A and 9C7A, were known as *indica*-specific subtypes and are consistent with a deletion in the ORF100 region of each plastid genome (Nakamura et al. 1998). These sequences and the deletion have not been detected in *japonica* cultivars. The CA repeats seem to have diverged after the deletion had occurred in *sativa* species. Other subtypes, 6C7A and 7C6A, were only detected in *japonica* cultivars. These sequences were tightly linked to non-deletion of the ORF100 region. Other combinations have not been found in *sativa* species. The CA repeats are not hyper-variable, like the nuclear microsatellites. Plant microsatellites in Gramineae species tend to show little or no variation when compared to the rice chloroplast (Ishii and McCouch 2000). This may depend on a mechanism of maternal inheritance of multiple plastids in these single cells. Thus, these plastid subtypes are now available to understand which cultivars are closely related, and how cultivars were introduced into particular countries.

Using the direct sequencing technique, we compared the linker sequences of numerous cultivars as landraces to known polymorphism in particular populations of this report. The comparison of the linker sequences revealed that Japanese lowland and upland rice populations consist of different plastid subtypes, respectively. We also compared them to foreign rice cultivars. The results indicated the relation of rice cultivars in different countries.

We also combined the genetic information obtained from the nuclear isozyme markers mentioned above. These nuclear and cytoplasmic data suggest how the Japanese upland population was formed, from where the population was introduced, what kinds of genetic composition they have, and how it differs from the Japanese lowland rice population. This information would be valuable for rice plant breeding and for an understanding of rice evolution.

Materials and methods

Plant materials

Fifty Japanese lowland cultivars and 80 Japanese upland cultivars were used. Their genetic characters have been described in Ishikawa et al. (1991, 1992, 1997). They were cultivated in lowland or upland fields in Japan. Thirty tropical and temperate *japonica* cultivars were examined for their nuclear and cytoplasmic genotypes, which have already been classified into these two groups by Oka (1953, 1958). Also, 135 Asian cultivars including Oka's tropical *japonica* cultivars (China, Indonesia, Laos, Philippines, Taiwan and Thailand) were used to compare their nuclear and cytoplasmic genotypes with those of Japanese cultivars.

Isozymes

Rice phosphogluconate dehydrogenase (PGD) is encoded by two genes. One of them, *Pgd1*, was scored by the procedure described by Ishikawa et al. (1991). Crude extracts of young seedlings were used for isozyme electrophoresis.

PCR and DNA sequencing

Total genomic DNA was isolated with the CTAB method from leaf tissues (100 mg). PCR amplification was performed with *Taq* DNA polymerase (Promega Co.), the company's recommended buffer, 0.2 mM of dNTPs in final concentration, and one pair of primers, 5Psk (CGCTCTAGAACTAGTGGATCAAAGATCTAG-ATTTCGTAAACAACATAGAGGAAGAA) and 3P (ATCTGCA-GCATTTAAAAGGGTCTGAGGTTGATCAT) (Nakamura et al. 1997). After 3 min of heat treatment at 94 °C, 45 cycles (98 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min) were used in the amplification, followed by 72 °C for 5 min as a post-treatment. From the PCR products, excess primers and free nucleotides were removed with the QIAGEN PCR purification kit, according to the company's recommendations. Then, 1 μg of PCR product was used as template DNA for sequencing. The LICOR 4200S sequencer was used with a sequence kit, the SequiTherm Excel II (EPICETRE TECHNOLOGIES Co.). An IRD41-labeled sk primer was employed for the reaction.

A deletion in the ORF 100 region was checked by the PCR reaction. Genotypes of chloroplast DNA were characterized by PCR amplification using primer set #3 (agtccactcagccatct) and #4 (ctcggccatcattttcttctttag) which amplify the ORF100 region in rice chloroplast DNA (Kanno et al. 1993). Most of the *indica* cultivars possess a 69-bp deletion in this region while most of *japonica* cultivars do not, and this region has been considered to be an effective marker for *indica–japonica* differentiation (Chen et al. 1993). After PCR amplification, PCR products were electrophoresed in a 1.5% agarose gel. *Takara Taq* DNA polymerase (Takara Co.) was used with the supplied reaction buffer, 0.2 mM of dNTPs, and 5 mM of $MgCl_2$. A high Mg concentration inhibited the amplification of additional bands for an unknown reason.

Results

Variation of plastid subtypes of Japanese lowland and upland cultivars

Forty nine lowland cultivars carried the subtype 6C7A, which is one of the *japonica*-specific subtypes like subtype 7C6A (Fig. 1, Table 1). One cultivar carried the *indica*-specific subtype 8C8A. Another plastid marker, a

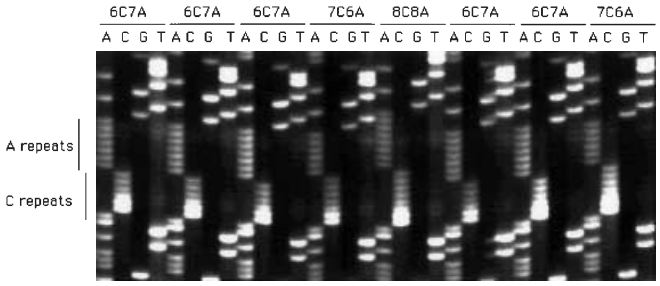

Fig. 1 Variation in plastid ID sequences found in Japanese rice cultivars. A portion of the PS-ID sequences is shown

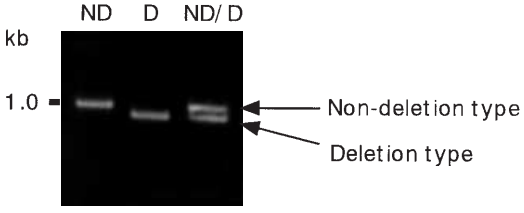

Fig. 2 Amplification of the ORF100 region. ND (Taichung 65, a lowland cultivar) and D (L32, a lowland cultivar) mean non-deletion-type and deletion-type plastids, respectively. *Arrows* indicate the respective bands. ND/D means a mixture of both amplified products

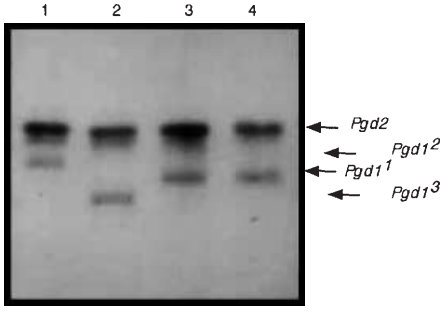

Fig. 3 Allelic polymorphism in *phosphogluconate dehydrogenase 1*(*Pgd1*)

PCR length polymorphism of ORFl00, was confirmed by PCR and subsequent electrophoresis of the amplified fragment. Then, the plastid subtype 8C8A was linked to a deletion of ORFl00 which is essential for the *indica*-type plastid (Fig. 2). However, the nuclear genotype examined for 17 isozyme loci (data not shown) was classified as a typical *japonica* type, like the other lowland cultivars examined. The cultivar with the subtype 8C8A can be explained as a hybrid between the *indica* and *japonica* cultivars. The subtype 7C6A was not observed in the lowland population. In contrast, the plastid in the upland rice population showed polymorphism of the PS-ID sequences. Thirty six of 80 upland cultivars carried the subtype 6C7A. The other 44 carried the subtype 7C6A. The subtype 8C8A was not found in the upland population.

Table 1 Variation of plastid subtypes and the occurence of *Pgd1* alleles in Japanese lowland and upland populations

Population	*Pdg1*	Plastid subtypes		
		6C7A	7C6A	8C8A
Lowland	1	49	0	1
Upland 1	1	3	4	0
Upland 2	2	33	40	0

Table 2 Distribution of plastid subtypes and the *Pgd1* genotype in temperate (Tm) and tropical (Tr) *japonica* cultivars

Population	Combinations of the plastid subtype-*Pgd1*			
	6C7A-allele1	7C6A-allele1	5C8A-allele2	6C7A-allele2
Tm	8	0	0	0
Tr	9	5	1	7

Isozyme genotypes and nuclear-cytoplasmic genotypes

Phosphogluconate dehydrogenase (PGD) is encoded by two loci in rice. *Pgd1* consists of three alleles (Fig. 3). One of them, *Pgd1*^{2}, is unique to tropical *japonica* cultivars (Table 2). This genotype could distinguish tropical *japonica* cultivars from temperate *japonica* cultivars, but not vice versa. *Pgd1*^{1} can be seen in both types of *japonica* cultivars. And the third one, *Pgd1*^{3}, is essential to the *indica* cultivars detected in Japan (data not shown). All temperate *japonica* cultivars carry *Pgd1*^{1} with the plastid subtype 6C7A, which is the same plastid subtype found in Japanese lowland cultivars. The upland-specific subtype, 7C6A, was then found in five of 14 tropical *japonica* cultivars carrying *Pgd1*^{1}. Seven of eight tropical *japonica* cultivars carrying *Pgd1*^{2} were of the subtype 6C7A. One carried the rare subtype 5C8A. Typical temperate *japonica* cultivars carry the subtype 6C7A and *Pgd1*^{1}.

Pgd1^2 is also specific to the Japanese upland rice population (Table 1). All but seven upland cultivars, with either subtype 6C7A or 7C6A, carried the upland-specific *Pgd1*^{2}. The seven cultivars carried the alternative allele, *Pgd1*^{1}. Three of the seven cultivars were of the subtype 6C7A and the remaining four cultivars were of the subtype 7C6A. Allelic frequencies at this locus were compared between two groups of upland cultivars carrying different plastid subtypes, and it was found that there was no difference (Table 1).

Among 135 cultivars from Southeast Asia including tropical and temperate *japonica* cultivars, one from the Philippines carried the rare subtype 5C8A mentioned above (Table 3). In Southeast Asian cultivars, the two plastid subtypes were also found. The subtype 7C6A was found in China, Indonesia, Laos and the Philippines, and Taiwan with another subtype 6C7A. In Thailand, only subtype 6C7A was found.

Table 3 Nuclear-cytoplasmic combination among *japonica* cultivars in Southeast Asian countries

Region	Total	6C7A-allele 1	6C7A-allele 2	7C6A-allele 1	7C6A-allele 2	5C8A-allele 2
China	63	59	3	1	0	0
Indonesia	26	9	14	1	2	0
Laos	18	17	0	1	0	0
Philippines	6	3	1	1	0	1
Taiwan	7	2	2	2	1	0
Thailand	15	15	0	0	0	0

A comparison of nuclear and cytoplasmic genotypes revealed three cultivars carrying a particular nuclear-cytoplasmic combination of *Pgd1*^{2} the subtype 7C6A, which is a unique combination in the Japanese upland rice population (Table 3). One came from Indonesia and the other two came from Taiwan.

Discussion

In *O. sativa* L., the number of C and A repeats in the linker sequences corresponds to the *indica* and *japonica* subspecies classifications. Two of the variants correspond to the *japonica* type and other three to the *indica* type. Two of the *japonica*-specific subtypes were found in Japanese upland cultivars, but only one in Japanese lowland cultivars with one exceptional case. One case found in L32 cultivars revealed the *indica*-specific subtype 8C8A. To confirm the origin of the plastid, a deletion of ORF100 in the plastid was examined by a PCR reaction. The 8C8A subtype was linked to a deletion of ORF100 in the *indica*-type plastid and such a deletion at ORF100 was not detected in other Japanese cultivars (data not shown). This *indica* plastid subtype is a very exceptional case because of the nuclear genotype which revealed a *japonica*-type allelic composition. Generally microsatellites are hyper-variable in the nuclear genome (reviewed in Ellegren 2000). Plastid microsatellites, however, are considerably stable (Ishii and McCouch 2000). It is hard to believe that the two mutations found in the plastid in the single cultivar occurred independently from the *indica* subspecies. Thus, the cultivar carrying the 8C8A subtype would be a descendant of a natural hybrid between the *japonica* and *indica* cultivars, but would not be a natural variant of the microsatellite. Once heterozygosity was generated in the nuclear genome it disappeared through many generations of self-pollination of the descendant. We can observe such naturally occurring hybrids in Laos, where both subspecies are still cultivated in single upland fields (Ishikawa et al., submitted) and some heterozygotes exist. The existence of heterozygotes is probably due to frequent cross-pollinations between the *indica* and *japonica* cultivars in the field. We have never detected heterozygotes in current genetic resources of the Japanese lowland and upland cultivars in comparison to that of Laotian genetic resources. This is because in Japan there are few *indica* strains as a pollen donor or a cytoplasmic donor (Ishikawa et al. 1991, 1992). In the past, however, several cultivars in the Daitou-mai group, genetically determined as *indica* cultivars (Ishikawa et al. 1992), were cultivated around *japonica* cultivars.

Gradually, these *indica* cultivars were purged from paddy fields to maintain uniformity of the lowland cultivars (Arashi 1961). Such *indica* cultivars could be pollen or cytoplasm donors against *japonica* cultivars. Since nuclear-cytoplasmic-substituted cultivars were generated in other countries they were then introduced into Japan in some frequency together with that of others. One would be the origin of the cultivar L32 carrying the *japonica* nuclear genotype with the plastid subtype 8C8A.

The variation found in the Japanese upland population would reflect the different origins of the Japanese lowland and upland populations. Differences between the two populations have also been documented for morphological and physiological traits regulated by the nuclear genome, as reviewed by Tsunoda (1997). Japanese upland rice cultivars have unique morphological characteristics. Many have thick wide leaves and a reduction of stomatal numbers. They also show a high field performance for blast fungus resistance. These characteristics are quite different from those of the lowland cultivars. Also, in genetic markers including 17 isozyme loci and five RFLP markers, the variation in nuclear genotypes of the Japanese upland cultivars was different from that in the lowland population (Ishikawa et al.1991, 1992, 1997). The most-remarkable distinction occurred in the genoype of locus *Pgd1*. More than 80% of the upland cultivars carried *Pgd1*^{2}, which has not been found in the lowland population.

In addition to these differences, Japanese upland cultivars are heterogeneous for several traits when compared with the lowland cultivars. Our previous report (Ishikawa et al. 1997), based on isozyme and RFLP markers, suggested that the upland cultivars are more polymorphic than the lowland cultivars, and have complex characters. Morphological and physiological characters showed the same tendency. Some upland cultivars show tropical *japonica* characters, such as in grain size, hypocotyl-elongation ability, and alkali degradation of the endosperm (Ishikawa et al. 1997). Other upland cultivars show characteristics intermediate between those of tropical and temperate *japonica* types. The PS-IDs enabled us to confirm assumptions on the origin of the Japanese upland rice population at the cytoplasmic level. In

the Japanese upland rice population, there are two plastid subtypes, 6C7A and 7C6A, in almost equal numbers. The plastid subtype 7C6A was found only in the upland rice population.

Variation in tropical *japonica* cultivars indicates that the Japanese upland rice population shares the same PS-ID sequence, 7C6A, with the tropical *japonica* cultivars. These data suggest that the Japanese upland population would partly originate from the tropical *japonica* cultivars. When nuclear genotypes were compared between the two upland groups carrying the plastid subtype 6C7A or 7C6A, the upland-specific allele *Pgd1*^{2} predominated in both groups. This result suggests that these two upland groups, carrying different plastid subtypes, originated from a single population which is not the same population from which the Japanese lowland population originated. The ancestral population of the Japanese upland rice population must have had *Pgd1*^{2} as the predominant genotype with both of the plastid subtypes. As tropical *japonica* cultivars carried the plastid subtype 7C6A or *Pgd1*^{2}, it is reasonable to assume that tropical *japonica* cultivars would be the ancestral population because the alternative *japonica* group has no cultivar carrying these nuclear and cytoplasmic genotypes. Alternatively, the plastid subtype 6C7A would be introduced from other populations the same as *Pgd1*^{1}, which might be derived by natural out-crossing with temperate *japonica* cultivars in east Asia other than from Japan. Japanese environmental conditions are not suitable for true tropical *japonica* cultivars. We suggest that the Japanese upland rice population received some genetic material from both tropical and temperate *japonica* cultivars. The offspring might be adapted to grow in Japonica upland fields according to heading date, pest resistance, fungus resistance and unknown factors.

To identify the original nuclear and maternal population of Japanese upland rice cultivars, we examined nuclear and cytoplasmic genotypes of tropical *japonica* cultivars from Southeast Asia. Cultivars with the plastid subtype 6C7A carried alleles 1 and 2 of *Pgd1*. Cultivars with subtype 7C6A also carried the same genotypes. Only one Indonesian and two Taiwanese cultivars carried the combination of *Pgd1*^{2} and the plastid subtype 7C6A. We found no such Chinese cultivars.

Sensho is one of the Japanese upland rice cultivars which was estimated to be introduced from China after the Japanese-Qing war about 100 years ago. However, we could not detect the same genotype as Sensho in Chinese cultivars. One explanation is that the materials we examined are not suitable for searching for related genetic variation to Japanese upland cultivars. The nuclear-cytoplasmic genotype found in Sensho was common with other Japanese upland cultivars. Thus, cultivars introduced from China into Japanese upland fields in the past were rare types of upland cultivars sharing almost the same genotypes with the Japanese upland cultivars. We detected cultivars carrying such a genotype in Indonesia and Taiwan, which would be closely related to the ancestors of the Japanese upland rice population. In Taiwan both types of *japonica* cultivars can be found. The boundary between these two types is in the tropic of cancer (Oka 1953). Ancestral cultivars of the Japanese upland population may outcross and exchange their genetic material between these two types of rice strains around the tropic of cancer. Tropical *japonica* cultivars are hardly grown in Japan because of their heading traits (data not shown). A part of the outcrossed progeny between tropical and temperate *japonica* cultivars may be the Japanese upland rice population, which is recombined for several cultivated habits in Japanese environmental conditions.

The Japanese upland rice population has unique nuclear genes and cytoplasm, compared to the Japanese lowland rice population. The relationship among Japanese and Southeast Asian upland cultivars offers an interesting scenario for the introduction of rice plants and genetic materials into Japan. These introductions should be different from that of the Japanese lowland rice population because of their different maternal origins, and from that of the *indica* cultivars found in lowland and upland rice populations. The idea of multi-dimensional introductions of rice plants into Japan would shed light on questions of where and how Japanese rice plants originated.

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