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ORIGINAL PAPER

Genetic analysis and molecular mapping of low amylose gene *du12*(t) in rice (*Oryza sativa* L.)

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Abstract

Key message **We obtained interesting results for genetic analysis and molecular mapping of the du12(t) gene.**

Abstract Control of the amylose content in rice is the major strategy for breeding rice with improved quality. In this study, we conducted genetic analysis and molecular mapping to identify the dull gene in the dull rice, Milyang262. A single recessive gene, tentatively designated as *du12*(t), was identified as the dull gene that leads to the low amylose character of Milyang262. To investigate the inheritance of *du12*(t), genetic analysis on an F_2 population derived from a cross between the gene carrier, Milyang262, and a moderate amylose content variety, Junam, was conducted. A segregation ratio of 3:1 ($\chi^2 = 1.71$, $p = 0.19$) was observed, suggesting that *du12*(t) is a single recessive factor that controls the dull character in Milyang262. Allelism tests confirmed that *du12*(t) is not allelic to other low amylose controlling genes, *wx* or *du1*. Recessive class analysis was performed to localize the *du12*(t) locus. Mapping of *du12*(t) was conducted on F_2 and F_3 populations of Baegokchal/Milyang262 cross. Linkage analysis of 120 F_2 plants revealed that RM6926 and RM3509 flank *du12*(t) at a 2.38-Mb region. To refine the *du12*(t) locus position, 986 F_2 and 289 F_3 additional normal plants were screened by the flanking markers. Twenty-six recombinant plants were identified and later genotyped with four additional adjacent markers located between RM6926 and RM3509. Finally, *du12*(t) was mapped to an 840-kb region on the distal region of the long arm of chromosome 6, delimited by SSR markers RM20662 and RM412, and co-segregated by RM3765 and RM176.

Abbreviations

du Dull
RM Rice microsatellite

Introduction

Over the past 3 decades, concern about rice quality has increased, resulting in rice quality becoming an important objective in breeding courses, along with yield and resistance traits. Since the amylose content of endosperm tissue is the major determinant of rice cooking and eating quality (Tan et al. 1999), controlling amylose content is the main strategy for breeding rice for quality improvement (Wang et al. 2009; Ni et al. 2011). Similar to the *wx* gene (Wang et al. 1995; Larkin and Park 2003), dull genes modify the expression of *Wx* gene (Zeng et al. 1997; Isshiki et al. 2000, 2008). Dull mutants with various levels of amylose content and starch properties have been obtained through mutagenesis induced by chemical and radioactive substances (Satoh and Omura 1981; Kaushik and Khush 1991; Koh and Heu 1997; Satoh et al. 2010). Due to the benefit of dull mutants in improving rice cooking and eating

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quality, rice breeders have been utilizing them to develop low amylose varieties or to reduce the amylose content of high amylose varieties (Okuno et al. 1993; Allahgholipour et al. 2006; Ando et al. 2010). To date, 13 genes controlling dull traits in rice have been reported. Satoh and Omura (1981) found some mutants with dull endosperm from cultivar Kinmaze through *N*-methyl-*N*-nitrosourea (MNU) treatment on fertilized egg cells. The chromosomal locations of the genes underlying the dull trait in the mutants, *du1*, *du2*, *du3*, *du4*, and *du5*, were observed by Yano et al. (1988). The *du1* gene shares 73.9 % identity with *Du1-Like* (*Du1L*) (Zeng et al. 1997). Koh and Heu (1997) used MNU mutagen on *Japonica* rice cultivar Hwacheong to generate two dull mutants with two recessive genes designated as *du-6a*(t) and *du-6b*(t), and one dominant gene, *Du-7*(t). The mutant lines had amylose content ranging from 5.9 to 9 %. Similarly, *duEM47* gene in a mutant line of cv. Kinmaze (EM47) was generated by MNU treatment, resulting in rice with a 1.9 % amylose content. Mutants harboring *du2120* and *du2035* genes were generated by ethyl methane sulfonate (EMS) treatment of cultivar Sasanishiki with amylose contents of 4.9 and 4.6 % amylose content, respectively (Kaushik and Khush 1991). Last, a dull gene named *Wx-mq* located on the waxy locus on chromosome 6 was identified in cv. Milky Queen (Sato et al. 2002). A mutant line named NM391 was obtained from gamma-irradiation-induced mutation. The mutant had dull characteristics with half the amylose content of its parent, Nihonmasari (Kinoshita and Kikuchi 1987). The dull gene, hereafter tentatively designated as *du12*(t), led to the development of several low amylose varieties, including Aya (Kunihiro et al. 1993), LGC soft (Shuichi and Yoishihiro 2004), and Ayahime (Kiuchi et al. 2009). However, its genetic characteristics have not been thoroughly investigated. Therefore, this study was conducted to elucidate the mode of inheritance of *du12*(t) and identify its genomic region.

Materials and methods

Plant materials and field trials

Several populations were developed from crosses between Milyang262 (Fig. 1S) and other parental cultivars (Junam, Milyang265, Baegokchal, and Baekjinju). All parental cultivars were *japonica* ssp. Milyang262 is a dull rice cultivar carrying the *du12*(t) gene derived from NM391, which was developed by crossing Junam and Chugoku173 (Fig. 1). All cross combinations were cultivated in the same time period. F_1 plants were generated in the summer of 2010 and cultivated in rice field in the summer of 2011. The harvested F_2 seeds were cultivated in the fall of 2011. The F_3 population of Baegokchal/Milyang262 was cultivated in the summer of 2012. F_1 plants were planted in paddy fields, while the succeeding generations were planted in greenhouses. Data on the heading date and temperature were not collected. Field trials were carried out at the National Institute of Crop Science, Rural Development Administration, Milyang, Korea.

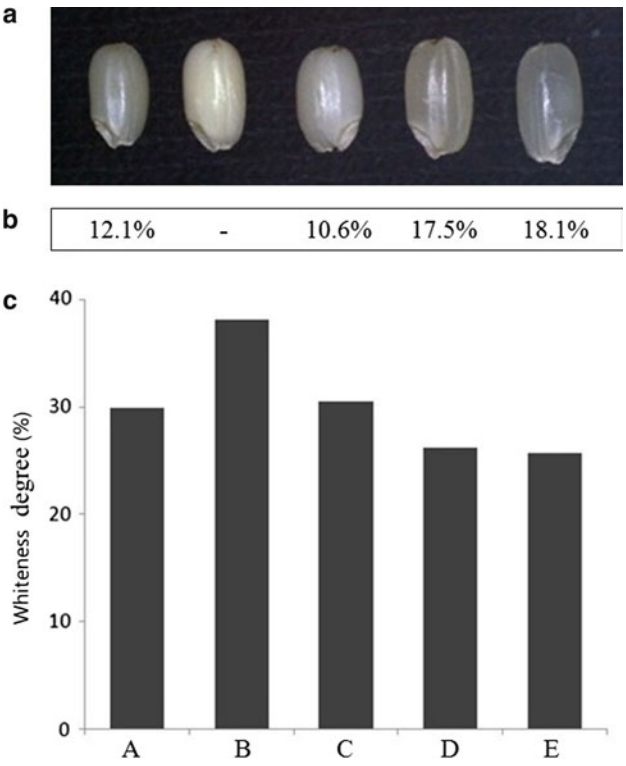

Fig. 1 Endosperm characteristics of parental varieties used in the current study. **a** Endosperm grain appearance; **b** amylose content (SE: 0.04–0.07); **c** degree of whiteness (SE: 0.05–0.16). *A* Milyang 262, *B* Baegokchal, *C* Baekjinju, *D* Junam, *E* Milyang265

Phenotypic evaluation

The phenotype of the endosperm character was evaluated on brown rice of seeds harvested at maturity. Harvested plants were threshed manually, followed by air drying until the moisture content reached ≤14 %. After manual dehulling, the translucency between individual grains was observed by visual inspection. Grains were considered normal for translucent endosperms, waxy for opaque white endosperms, and dull for hazy white endosperms that fell between opaque and translucent. To verify the differing endosperm characters, the amylose content and degree of whiteness of the parent cultivars were measured. The distribution of amylose content and degree of whiteness among mapping populations could not be measured because the seeds were treated as individuals and had to be grown. A Kett digital whiteness meter, model C-300-3 (Kett Electric Laboratory, Tokyo, Japan), calibrated with a magnesium

oxide standard plate with a whiteness reading of 87.4 % was used to measure the degree of whiteness. The measurement was conducted in triplicate by following the manufacturer's instructions. Amylose content analysis was conducted according to the procedure described by Juliano (1971). The optical density at 620 nm was measured using a T80 + UV/VIS spectrometer (PG Instruments Ltd., Leicestershire, UK).

Inheritance mode of *du12*(t) and tests for allelism with *du1* and *wx*

An F_2 population derived from a Junam/Milyang262 cross was used to determine the inheritance mode of *du12*(t). Allelism tests were conducted to verify the independent inheritance of *du12*(t) to *wx* gene and another dull gene, *du1*. Milyang262 was crossed with Baegokchal and Baekjinju, which harbor *wx* and *du1*, respectively. Segregations of endosperm characters in genetic analysis and the allelism test were observed on 200 F_2 seeds. Chi-square (χ^2) tests were used to evaluate the goodness of fit of the observed and expected segregation ratio in genetic analysis and allelism tests.

DNA extraction and genotyping

Genomic DNA was extracted from fresh leaf tissues of individual plants at seedling stage using the cetyltrimethylammonium bromide (CTAB) procedure with some modifications. Briefly, 3-mg leaf samples were cut into small pieces and then placed into 2-ml tubes with three tungsten beads. The tubes were then soaked in liquid nitrogen for approximately 60 s, after which they were vortexed. A mixture of 350 µl extraction buffer and 2 µl proteinase K was subsequently added, and the samples were then incubated at 37 °C for 15 min. Next, 350 µl of 2 % CTAB was added, and the samples were incubated at 65 °C for 30 min, after which 500 µl of phenol:chloroform:isoamyl alcohol (PCI) 25:24:1 was added, and the samples were shaken using an inverter for 10 min. The samples were subsequently centrifugated at 13,000 rpm for 10 min, after which a 500-µl aliquot of the upper phase (aqueous) was collected from the tube and transferred into a new 1.5-ml tube. A DNA pellet was obtained after precipitation by 500 µl isopropanol and centrifugation at 13,000 rpm. The extracted DNA was stored in 0.1× Tris-EDTA buffer. Polymerase chain reaction was carried out in a GeneAmp® PCR system 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles of amplification (denaturation at 94.5 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min), and final extension at 72 °C for 7 min. The reaction mixture was a total of 20 µl that consisted of 50 ng DNA template, 1× PCR buffer, 0.8 µM dNTP, 5 pmol of each forward and reverse primer, and 0.1 U Prime™Taq polymerase G1000 (GeNet Bio, Daejeon, Korea). The amplicons were separated on 4 % polyacrylamide gel in 0.5× TBE buffer or on 3 % agarose gel depending on the length of the amplified fragments, after which they were visualized using Gelstar™ nucleic acid stain (Lonza, Rockland, ME, USA) and SYBR® safe DNA gel stain (Invitrogen, Eugene, OR, USA), respectively.

Recessive class analysis

DNA polymorphisms between parents were assessed by a set of 142 randomly selected pairs of simple sequence repeats (SSR) markers (http://www.gramene.org) distributed in all 12 chromosomes. The average distance between SSR markers was 2.46 Mb. To determine the chromosomal position of *du12*(t), we performed recessive class analysis (Zhang et al. 1994) with some modifications. Instead of making a bulked DNA pool, this was accomplished by examining eight or nine recessive (dull) F_2 individuals from Junam/Milyang262, Baegokchal/Milyang262, and Milyang265/Milyang262 crosses with the respective, previously identified polymorphic SSR markers. The markers producing DNA bands that matched the phenotype were regarded as the candidate linked markers for the *du12*(t) locus. Subsequently, to ascertain the candidate *du12*(t) linked markers, they were genotyped with 30 F_2 individuals.

Fine mapping of the *du12*(t) locus

Three population groups derived from the Baegokchal/Milyang262 cross were used as the mapping material (Fig. 2). Linkage analysis was carried out in an F_2 population consisting of 89 normal and 31 dull individuals using SSR markers located near the putative linked marker. To refine the position of *du12*(t), a second F_2 population group consisting of 986 normal individuals was used. Two flanking markers of the *du12*(t) gene identified in linkage analysis were used to identify recombinant plants. The recombinant plants were later genotyped by additional SSR markers located between the flanking markers. Additionally, we selected normal individuals by segregating $F_{2:3}$ grains of each key recombinant F_2 plant and generated an F_3 population consisting of 289 normal F_3 plants. The screening and genotyping in the F_3 populations were carried out with the same method as in the F_2 population. The molecular map was constructed by analyzing the recombination events occurring on either side of the *du12*(t) locus. The physical position of SSR markers was based on Gramene Annotated Nipponbare Sequence 2009 (http://www.gramene.org).

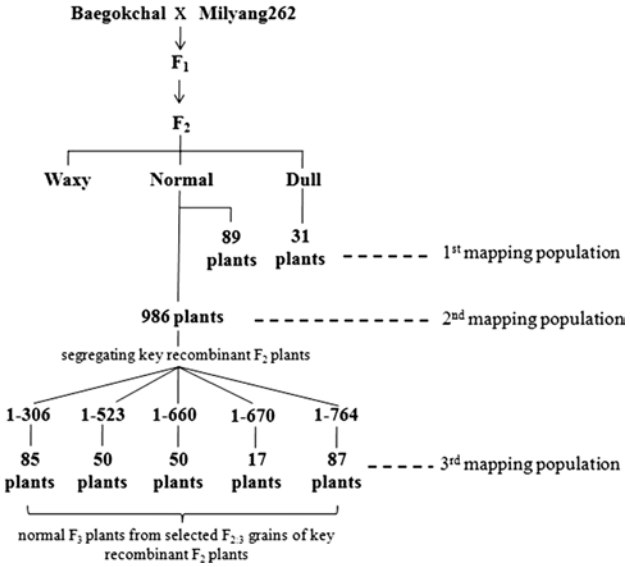

Fig. 2 Plant materials used for mapping *du12*(t). Genetic analysis was carried out using the first mapping populations, while further fine mapping was carried out using the second and third populations

Results

Phenotypic evaluation

The endosperm characters of the parental cultivars fell into three distinct classes: normal, dull, and waxy. The endosperms showed different degrees of translucency because of the differing amylose contents (Fig. 1). Normal endosperm was observed in the moderate amylose content endosperm found in Junam and Milyang265, while dull endosperms were observed in those of Milyang262 and Baekjinju, which have low amylose content. Baegokchal was found to have opaque endosperm reflecting its waxy nature.

Inheritance mode of *du12*(t)

The F_1 grains of Baegokchal/Milyang262, Junam/Milyang262, and Milyang265/Milyang262 crosses were all normal, indicating that the dull trait in Milyang262 was controlled by recessive gene(s). Genetic analysis in the F_2 population of Junam/Milyang262 demonstrated a segregation pattern of 3 normal:1 dull ($\chi^2 = 1.71$, $p = 0.19$), indicating a single recessive mode of *du12*(t) in controlling the dull character in Milyang262 (Table 1).

Allelism test of *du12*(t) against *wx* and *du1*

Allelism tests suggested that *du12*(t) is not allelic to *wx* or *du1* (Table 1). In the F_2 population of the Baegokchal/Milyang262 cross, endosperm character segregated into a ratio of 9 normal:3 dull:4 waxy. The combination of recessive-recessive homozygous genotype *wxwxdu12*(t)*du12*(t) was expressed as waxy in phenotype and thus modified the Mendelian ratio from 9:3:3:1 to 9:3:4. The segregation pattern in the Baekjinju/Milyang262 cross also fits the expected ratio of 9 normal:7 dull. In this case, due to the similar phenotype of *du1*, *du12*(t), and their combination, the phenotype ratio was 9 normal:7 (3 + 3 + 1) dull.

Recessive class analysis of dull character in Milyang262

The polymorphism rate between Milyang262 and Baekjinju (33.01 %) was higher than those for Junam (17.61 %), Milyang265 (32.39 %), or Baegokchal (26.06 %) (Table 1S). According to the result of the genetic analysis that *du12*(t) controls the dull character in single recessive mode, the recessive class analysis (Zhang et al. 1994) was employed to determine the location of *du12*(t). This was accomplished by examining eight to nine F_2 dull individuals of Junam/Milyang262, Baegokchal/Milyang262, and Milyang265/Milyang262 crosses by the respective, previously identified polymorphic markers. The dull individuals were assumed to be homozygous for the Milyang262 allele at the targeted *du12*(t) locus. Three markers on the long arm of chromosome 6, RM6818 (16.58 Mb), RM6458 (27.56 Mb), and RM3509 (30.97 Mb), were indicated to be linked to *du12*(t) and were further tested with 30 individuals (data not shown). RM3509 was later regarded to be closely linked to the *du12*(t) locus as it resulted in DNA bands that matched the phenotype.

Fine mapping of the *du12*(t) locus

Populations derived from the Baegokchal/Milyang262 cross were used as the mapping materials. The F_2 progenies of this population segregated into three classes of

Table 1 Segregations of endosperm characters in F_2 populations of cv. Milyang262 crossed with three different parents used for genetic analysis and allelism tests

Cross	Brown rice phenotype of F_2 seeds			Exp. ratio	χ^2	*p* Value
	Normal	Dull	Waxy			
Junam/Milyang262	158	42	–	3:1	1.71	0.19
Backogchal/Milyang262	108	48	44	9:3:4	3.84	0.15
Baekjinju/Milyang262	124	76	–	9:7	2.69	0.10

endosperm characters (normal, dull, and waxy). The normal phenotype consisted of dominant and hetero genotypes, which are *Du12*(t)*Du12*(t) and *Du12*(t)*du12*(t), *du12*(t)*Du12*(t), respectively, while the dull phenotype had only a homozygous genotype, *du12*(t)*du12*(t). In the case of the waxy phenotype, it could also have the *du12*(t) allele, but it was difficult to distinguish waxy individuals without a homozygous *du12*(t) allele (*wxwxDu12*(t)*Du12*(t), *wxwxDu12*(t)*du12*(t)) and with a homozygous *du12*(t) allele (*wxwxdu12*(t)*du12*(t)). Therefore, waxy individuals were not used as mapping material.

Three populations derived from the Baegokchal/Milyang262 cross were generated to map *du12*(t) (Fig. 2). Initial mapping of *du12*(t) was conducted on 120 F_2 plants consisting of 89 normal and 31 dull individuals. Six additional polymorphic markers around RM3509 (RM20590, RM400, RM1370, RM6926, RM6811, RM412) were used for linkage analysis (Table 2). The *du12*(t) locus was mapped to a 2.38-Mb region between RM6926 (28.59 Mb) and RM3509 (30.97 Mb) and tightly linked with RM6811 and RM412.

To refine the position of *du12*(t), the second group of the F_2 population was used. The flanking markers RM6926 and RM3509 were first used to screen 986 normal individuals. Eight recombinants were identified by RM6926, while 18 recombinants were identified by RM3509 (Fig. 3). We later surveyed the polymorphisms of 17 SSR markers located between RM6926 and RM3509, of which four markers (RM20662, RM3765, RM176, RM103) were found to be polymorphic. The additional markers were further used to examine the 25 previously identified recombinants. As a result, RM103 detected ten recombination events, while three recombination events were detected by RM412.

Later, the third population was generated from normal $F_{2:3}$ grains of the key recombinant F_2 plants. As many as 289 normal F_3 individuals were selected from the segregating $F_{2:3}$ grains and were later grown. One recombination event each between *du12*(t) and RM6811 and RM20662

Table 2 Linkage analysis of *du12*(t) locus and SSR markers on chromosome 6 in the F_2 population derived from the Baegokchal/Milyang262 cross

Locus		AABB/ AABb	AA bb	Aa BB	Aa Bb	Aa bb	Aa BB	Aa Bb	Aa bb	Rec. value (%)	Location (Mb)
A	B										
RM20590	*du12*(t)	47	0	40	0	2	2	0	29	3.33	28.02
RM400	*du12*(t)	46	0	41	0	1	2	0	30	2.50	28.43
RM1370	*du12*(t)	45	0	42	0	1	2	0	30	2.50	28.53
RM6926	*du12*(t)	46	0	41	0	0	2	0	31	1.67	28.59
RM6811	*du12*(t)	46	0	43	0	0	0	0	31	0	29.23
RM412	*du12*(t)	46	0	43	0	0	0	0	31	0	30.33
RM3509	*du12*(t)	44	0	44	0	0	1	0	31	0.83	30.97

A/a represents the SSR marker genotype. AA: homozygous for the Baegokchal allele, Aa: heterozygous, aa: homozygous for the Milyang262 allele. B/b represents the phenotype for *du12*(t). BB/Bb:normal/translucent, bb: dull

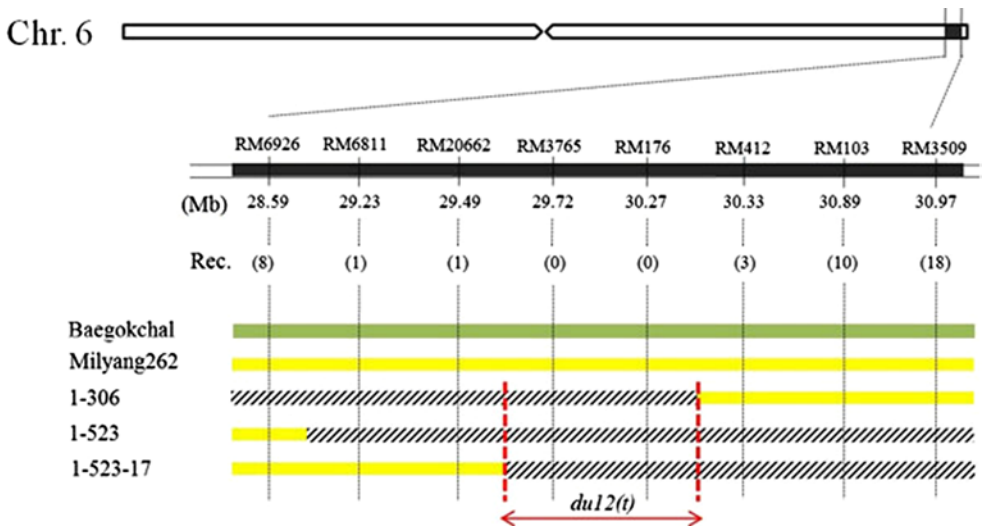

Fig. 3 Physical map of the chromosomal region that contains the *du12*(t) gene

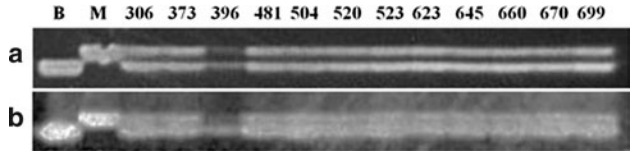

Fig. 4 Co-segregation of markers RM3765 (**a**) and RM176 (**b**) with *du12*(t). *B* Baegokchal, *M* Milyang262. *Lane 3–14* key recombinant plants showing heterozygosity at the *du12*(t) locus

was detected in an F_3 plant, 1-523-17. These results thus defined the *du12*(t) locus as an 840-kb region delimited by RM20662 and RM412. No recombination occurred between *du12*(t) with markers RM3765 and RM176, suggesting tight linkages between the markers and *du12*(t). Figure 4 demonstrates the co-segregation of RM3765 and RM176 with *du12*(t). To verify the fine mapping result, grains of all recombinants plants (25 F_2 plants and 1 F_3 plant) were observed for their phenotype, in which their endosperm character was segregating as expected.

Discussion

The dull trait in rice leads to low amylose content of its endosperm. Here, we report a novel dull locus in rice designated as *du12*(t). *du12*(t) was mapped to an 840-kb region in the distal region of the long arm of chromosome 6, delimited by SSR markers RM20662 and RM412. This result provides information about a new location of the dull locus in rice. Previous studies reported different locations of dull genes (Zeng et al. 1997; Yano et al. 1988; Sato et al. 2002).

Genetic analysis indicated that *du12*(t) segregates in a monogenic pattern, which denies any possibility of allelic relationship with other gene(s) in controlling dull character in Milyang262, including *duEM47* and *du2035*, which also located on chromosome 6 (Kaushik and Khush 1991). The physical locations of *duEM47* and *du2035* have not been investigated yet. However, it is unlikely that *du12*(t) is the same gene as *duEM47* or *du2035* because of the different phenotypes, types of mutagenesis, and germplasm origin. In this study, *du12*(t) lowers the amylose content in Milyang262 to 12.1 %, while Kaushik and Khush (1991) reported amylose contents of 4.9 and 4.6 % for *du2120* and *du2035*, respectively. *du12*(t) originated from radiation mutagenesis on cv. Nihonmasari, while *duEM47* and *du2035* were identified after chemical mutagenesis on cv. Kinmaze and cv. Sasanishiki.

du12(t) controls the dull character in single recessive mode, similar to other previously reported dull genes (Yano et al. 1988; Kumar and Khush 1988), with *du6*(t), *du6b*(t), and *Du7*(t) as exceptions (Koh and Heu 1997). The recessive nature of *du12*(t) was previously discovered, although the segregation was anomalous (Kinoshita and Kikuchi 1987). The abnormality could possibly be attributed to the difference of subspecies-specific *Wx* alleles between parents (Sano et al. 1986), which causes the dull gene to induce only a single gene effect, which is less significant in the genetic isolation of a gene with major effect. Dull genes have been reported to alter the Wx^b gene expression, which predominantly exists in *japonica* ssp. (Isshiki et al. 2000; Zeng et al. 1997). Dung et al. (2000) also reported different segregation patterns between *du2-2*Wx^a and *du2-2*Wx^b, suggesting different interaction between the dull gene and the *Wx* alleles.

Allelism tests confirmed the independent inheritance of *du12*(t) to other low amylose controlling genes, *wx* and *du1*. However, it is also noted that an additive effect (Kumar and Khush 1986) might be present. Ranges of the translucency level among dull F_2 grains in Baekjinju/Milyang262 cross and waxy F_2 grains in Baegokchal/Milyang262 cross were arguably apparent. Combination of low amylose genes such as in genotypes *du12*(t)*du1* and *du12*(t)*wx* might have induced lower amylose content than sole *du12*(t), *du1*, or *wx* genes do.

An attempt to find a candidate gene for *du12*(t) was made. By assuming the possibility of similar molecular function between *du12*(t) and *du1* (Zeng et al. 1997), gene Os06g49740.1, which encodes a putative pre-mRNA splicing factor syf2 (Rice Functional Genomic Express Database, SIGnAL), located at 30.09 Mb, was considered a potential candidate gene. However, sequence analysis did not show any difference between Milyang262 and Baegokchal in this region. Nevertheless, the finding of the *du12*(t) locus could lead to narrowing down of the *du12*(t) region by using a succeeding generation of the current mapping population, map-based cloning, and subsequently, characterization of the molecular function of the gene. In addition, the availability of the co-segregating markers could be used in marker-assisted breeding for rice quality improvement.

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Conflict of interest None.

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