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ORIGINAL PAPER

Distribution and mapping of an active autonomous *aDart* element responsible for mobilizing nonautonomous *nDart1* transposons in cultivated rice varieties

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Abstract An endogenous 0.6-kb rice DNA transposon, *nDart1*, has been identified as a causative element of a spontaneous mutable *virescent* allele *pyl-v* conferring pale-yellow leaves with dark-green sectors in the seedlings, due to somatic excision of *nDart1* integrated into the *OsClpP5* gene encoding the nuclear-coded chloroplast protease. As the transposition of *nDart1* depends on the presence of an active autonomous *aDart* element in the genome, the plants exhibiting the leaf variegation carry the active *aDart* element. As several mutable alleles caused by *nDart1* insertions have subsequently been identified, *nDart1*-promoted gene tagging has been proven to be an effective system. At present, the *nDart/aDart* system appears to be the only endogenous rice DNA transposon system whose transposition activity can be controlled under natural growth conditions without any artificial treatments, including tissue cultures. To apply the *nDart/aDart* tagging system in various cultivated rice varieties, we explored the presence and distribution of an active autonomous *aDart* element in 19 temperate japonica, 30 tropical japonica, and 51 indica varieties. Only eight temperate japonica varieties were found to bear a single copy of an active *aDart* element, and no *aDart* activity could be detected in the indica varieties examined. Six of seven japonica varieties appear to carry the active *aDart* element at the identical site on chromosome 6, whereas the remaining one contains *aDart* on chromosome 5. Leaf variegations in the plants with the mutable *pyl-v* allele and the excision frequencies of endogenous *nDart1* elements indicated that the *aDart* element on chromosome 6 is more active than that on chromosome 5. The findings described here are an important step in the development of a new and efficient *nDart1*-promoted gene-tagging system in various rice cultivars.

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Introduction

With the availability of the 389-Mb rice genome sequence (International Rice Genome Sequencing Project 2005), the gene number of rice is predicted to be either ~32,000 (The Rice Annotation Project 2007, see also http://rapdb.dna.affrc.go.jp/) or ~41,000 (TIGR Rice Genome Pseudomolecules release 5; http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules/info.shtml). To elucidate the function of these putative genes, various mutants, including random insertional mutants with foreign elements, such as T-DNA or maize DNA transposons, and the endogenous retrotransposon *Tos17* have been generated (Hirochika et al. 2004; Zhu et al. 2007). A potential obstacle to these insertional mutants may be the concomitant occurrence of somaclonal variations (Larkin and Scowcroft 1981) because tissue cultures are necessary to either introduce these foreign elements into rice calli or activate dormant *Tos17* in the genome. Indeed, the tagging frequency of *Tos17* was reported to be only 5–10% because of the high occurrence of somaclonal variations (Hirochika 2001). Recently, an active 0.6-kb endogenous DNA transposon,

nDart1 (*nonautonomous DNA-based active rice transposon one*), belonging to the *hAT* superfamily, was identified as a causative element of a spontaneous mutable *virescent* allele, *pyl-v* (*pale-yellow leaf-variegated*), which confers pale-yellow leaves with dark-green sectors in the seedlings (Tsugane et al. 2006). The *pyl-v* allele is caused by the disruption of the nuclear-coded chloroplast protease gene, *OsClpP5*, due to insertion of one of the *nDart1* elements, named *nDart1-0*. In the presence of an active autonomous *aDart* element, somatic excision of *nDart1-0* from *OsClpP5* results in the pyl-v leaf-variegation phenotype; a dark-green sector comprises somatically reverted cells resulting from the *nDart1-0* excision, while a pale-yellow background consists of cells having *nDart1-0* inserted into *OsClpP5* in the homozygous condition. Plants containing the *pyl-v* allele without an active *aDart* element exhibit pale-yellow leaves without variegation, which is termed as the pyl-stb (pale-yellow leaf-stable) phenotype. It has also been shown that the transposition of *nDart1-0* can be induced by crossing with a temperate japonica line, H-126, containing an active *aDart* element, and that the *nDart1* insertions can be stabilized by segregating the *aDart* element under natural growth conditions. While japonica lines, including Nipponbare, carry multiple copies of epigenetically silenced inactive *iDart* elements, plants exhibiting the pyl-v leaf-variegation phenotype contain only one copy of the active *aDart* element (Tsugane et al. 2006). At present, the *nDart/aDart* system appears to be the only endogenous rice DNA transposon system whose transposition activity can be controlled under natural growth conditions without any artificial treatments, including tissue cultures. This feature of the *nDart/aDart* system may hold a considerable advantage over other gene-tagging systems in rice, because no somaclonal variations due to tissue cultures are expected to occur. Moreover, several mutable alleles caused by the insertion of *nDart1*, including *thumbelina-mutable*, which confers the mutable gibberellin-insensitive dwarf phenotype, have already been identified (Fujino et al. 2005; Tsugane et al. 2006; M. Maekawa, K. Takagi, and K. Tsugane, unpublished), indicating that our *nDart1*-promoted gene-tagging system is effective.

As a first step to apply the *nDart/aDart* system for efficient gene tagging in various rice varieties, we explored the presence and distribution of an active autonomous *aDart* element in various cultivated rice plants; 19 varieties of temperate japonica, 30 of tropical japonica, and 51 of indica were examined. Only eight temperate japonica varieties were found to bear a single copy of an active autonomous *aDart* element that can mobilize *nDart1-0* residing within the *OsClpP5* gene. Interestingly, seven varieties, consisting of one and six varieties originated from Japan and China, respectively, conferred leaf variegation phenotypes that were indistinguishable from the previously characterized T-65 *pyl-v* line (Tsugane et al. 2006), whereas the remaining one variety originated from Japan showed a clearly different variegation pattern. Subsequently, analysis suggested that the *aDart* elements in the major seven varieties are likely to be identical to the *aDart* element carried by the authentic T-65 *pyl-v* line on chromosome 6 and that the *aDart* element in the minor Japanese variety is a new active autonomous element located on chromosome 5. These findings would facilitate the development of a new and efficient gene-tagging system in rice and also shed light on an evolutionary aspect of active autonomous elements in the *nDart/aDart* system.

Materials and methods

Detection of an active *aDart* element by crossing with the tester *pyl-stb* line

The 100 varieties to be tested for the presence of *aDart* activity comprise 19 temperate japonica, 30 tropical japonica, and 51 indica varieties (Suppl. Table 1); among them are 52 varieties from the World Rice Collection (WRC) (Kojima et al. 2005) together with 20, 11, 10, 3, 1, and 3 varieties originated in Japan, China, India, Indonesia, the Philippines, and Europe, respectively. Although WRC consists of 69 varieties selected to cover 90% of the diversity of all RFLP polymorphisms (http://www.gene.affrc.go.jp/plant/core_collections-wrc01.html), five varieties were not provided by the NIAS Genebank, and 12 varieties could not be used for crossing because of very late heading or insect injury. The rice varieties to be examined were crossed with the T-65 *pyl-stb* tester line as a female parent to examine whether the plant in question bears an active *aDart* element. The tester line, T-65 *pyl-stb*, which exhibits only pale-yellow leaves without variegations, has *nDart1-0* integrated into the *OsClpP5* gene on chromosome 3 and carries no active *aDart* element (Tsugane et al. 2006). In the F2 population from a cross between the *pyl-stb* tester line and a variety containing one copy of an active *aDart* element, therefore, the occurrence of mutants displaying the leaf-variegated pyl-v phenotype to stable *pyl-stb* plants will have a ratio of 3:1. However, no such pyl-v mutants will be segregated at the seedling stage in the F2 population if the variety under examination bears no active *aDart* element.

Nucleic acid procedures and mapping of an active *aDart* element

All nucleic acid procedures, including the preparation of genomic DNA, RNA, PCR, and RT-PCR amplification, are as previously described (Tsugane et al. 2006; Terada et al. 2007). To assess an active *aDart* element, we first examined

whether *aDart* resides at or near the integration site of the active autonomous element, which corresponds to *iDart1-27* in Nipponbare, on chromosome 6 in the previously characterized *pyl-v* line (Tsugane et al. 2006; M. Maekawa, unpublished data). We employed *pyl-stb* segregants containing no active *aDart* element in the F2 populations from crosses between the varieties to be tested and the *pyl-stb* tester line and analyzed whether the *iDart1-27*-containing region on chromosome 6 is derived from the *pyl-stb* tester line by using the simple sequence repeat (SSR) markers RM5509 and RM6811 (McCouch et al. 2002), which are located near *iDart1-27* on chromosome 6 (see Fig. 2b). PCR amplification to detect these polymorphisms was performed with appropriate primers: initial denaturation at 95°C for 7 min, 30 cycles of denaturation at 95°C for 45 s, and annealing and extension at 55°C for 1 min and at 72°C for 2 min, respectively.

Because no apparent linkage between *aDart* in Nakateaikoku and *iDart1-27* represented by the SSR markers RM5509 and RM6811 was detected and because the *aDart* activity in Nakateaikoku appeared to differ from the *iDart1-27*-related *aDart* activities in other varieties, we tried to map the active *aDart* element in Nakateaikoku by using a pyl-v segregant obtained from the F2 population from a cross between Nakateaikoku and the *pyl-stb* tester line. A pyl-v segregant was crossed with Kasalath, which shows no *aDart* activity (Tsugane et al. 2006), and pyl-stb segregants in the F2 population of the cross between the pyl-v segregant and Kasalath were selected at their seedling stage. The genomic DNAs extracted from the pyl-stb segregants were subjected to mapping of *aDart* using 57 SSR markers for genome-wide coverage (Maekawa et al. 2005).

To detect the presence or absence of an *iDart1-26*-related element, PCR amplification was performed with either the primers 1-26-4F (5′-TTTCATCAGCCGTGCCTAAC-3′) and 1-26-4R (5′-AACGCGTAGTCTCGAGGAAG-3′) or the primers 1-26-1F (5′-GCATTCCTAAGGGCTGTTCA-3′) and 1-26-1R (5′-AACACCAGGGGAGGAGGTAG-3′). Similarly, the primers 1-27-3F (5′-TTTCATCAGCCGTGCCTAAC-3′) and 1-27-3R (5′-CAAAAGAATTTTGCCTCACG-3′) for PCR analysis were used to detect the presence of an *iDart1-27*-related element. PCR analysis with appropriate primers was also employed to examine whether *nDart1* elements can be found at identical sites in Nipponbare (Tsugane et al. 2006).

To characterize the 737-bp fragments consisting of 54-bp 5′-UTR and 683-bp 5′ coding regions and derived from expressed transposase transcripts of the *iDart1*-related elements, RT-PCR-amplified fragments were isolated as described previously (Terada et al. 2007). After having synthesized first-strand cDNAs, subsequent PCR amplification was performed with primers Dart-F1 (5′-CGTAGTTCAACAGTTTGATCGCAGAGG-3′) and Dart-R1 (5′-AGTACATGTACTAAACAGTTCCTTAAG-3′): initial denaturation at 98°C for 1 min, 8 cycles of denaturation at 98°C for 10 s, annealing at 74°C for 10 s, followed by decreasing 1°C per cycle, and extension at 72°C for 20 s, and then 27 cycles of denaturation at 98°C for 10 s, annealing at 65°C for 10 s, and extension at 72°C for 20 s. To analyze the distribution of the transcripts from *iDart1*-related elements, an individual RT-PCR-amplified product was cloned and sequenced; *iDart1*-related elements producing transcripts were identified by comparing polymorphisms in the sequenced 737-bp region. For an internal control, the 438-bp segments derived from transcripts of the four constitutively expressed genes encoding elongation factor 1α proteins (Os03g0177400, Os03g0177500, Os03g0177900, and Os03g0178000) were amplified with primers eEF1α-F1 (5′-GCCGAGCGTGAGAGAGGTATC-3′) and eEF1α-R1 (5′- CCAGTCAAGGTTGGTGGACCT-3′): initial denaturation at 94°C for 1 min, 27 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min.

Results

Detection of an active *aDart* element in eight varieties

Of 100 varieties examined by crossing with the T-65 *pyl-stb* tester line, eight cultivars, two of Japanese and six of Chinese origin, gave variegated pyl-v segregants in the F2 populations (Table 1). All of them belong to temperate japonica, and no indica varieties examined were shown to bear any active *aDart* element. The segregation frequencies of *pyl-v* plants to total *pyl* plants were found to be 72–84%, which fitted well with the expected 3:1 segregation ratio, indicating that each of them carries a single active *aDart* element that can mobilize *nDart1-0* residing at *OsClpP5*. Seven of eight varieties gave pyl-v segregants exhibiting leaf variegations, which are very similar to those observed in the authentic *pyl-v* plant; typical examples are shown in Fig. 1. Of these, six varieties originated from China, Guangxi-3 to -8, display very similar phenotypes (data not shown), implying that they may be closely related cultivars, although their origins remain obscure. The remaining one, a Japanese cultivar, Nakateaikoku, produces pyl-v segregants that display leaf variegations that are much smaller than those observed in the other pyl-v plants, including the authentic T-65 *pyl-v* line. Such differences in leaf variegation patterns can be observed not only in their second leaves but also in their third leaves (Fig. 1). Thus, the *aDart* activity in Nakateaikoku appears to differ from those in seven other varieties in Table 1.

As the active *aDart* element in the authentic *pyl-v* line was found to correspond with *iDart1-27* of 3,574 bp in

Table 1 Segregation of pyl-v plants in the F2 populations from crosses between the rice varieties to be tested and the *pyl-stb* tester line

Crossed variety	Origin	F2 segregation			Total	pyl-v plants/total of pyl plants (%)	χ^2 for 3:1[a]
		Normal	pyl-stb	pyl-v			
Nakateaikoku	Japan	101	8	43	152	84.3	2.36[b]
Guangxi-3	China	164	22	71	257	76.3	0.09[b]
Guangxi-4	China	182	22	110	314	83.3	4.89[c]
Guangxi-5	China	172	22	70	264	76.1	0.06[b]
Guangxi-6	China	171	19	76	266	80.0	1.27[b]
Guangxi-7	China	154	32	90	276	73.8	0.10[b]
Guangxi-8	China	188	30	111	329	78.7	1.04[b]
Yaenomidori	Japan	105	11	28	144	71.8	0.21[b]

[a] Segregation of 3 pyl-v and 1 ply-stb

[b] Not significant

[c] Significant at the 5% level

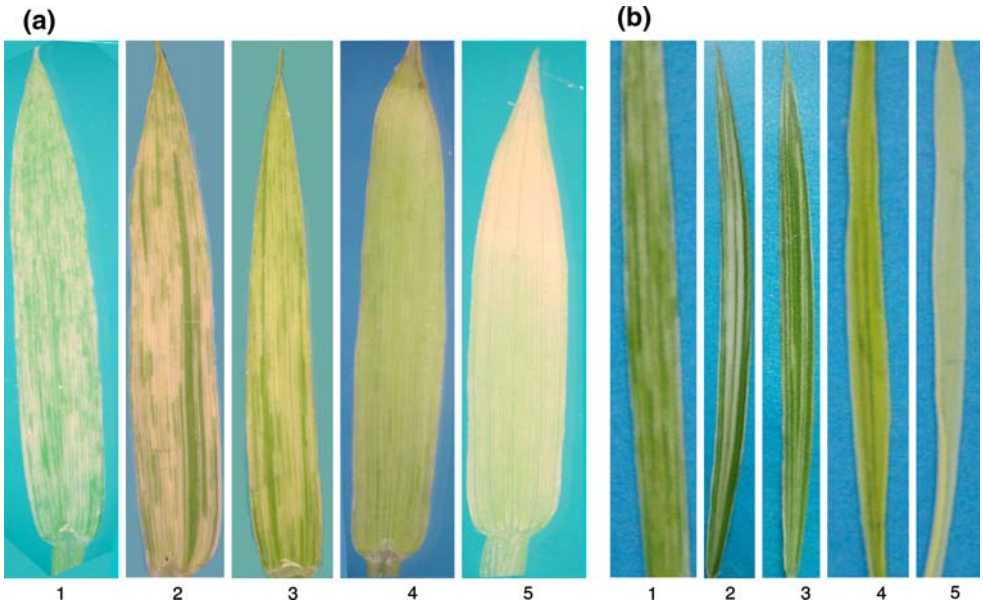

Fig. 1 Variegated second (**a**) and third (**b**) leaves of pyl-v and pyl-stb plants. The pyl-v plants with *aDart* from Guangxi, Yaenomidori, and Nakateaikoku were found in the F2 populations of the crosses between T-65 pyl-stb x Guangxi, Yaenomidori, and Nakateaikoku, respectively. (*1*), authentic T-65 *pyl-v* plant; (*2*), a pyl-v plant with *aDart* from Guangxi-3; (*3*), a pyl-v plant with *aDart* from Yaenomidori; (*4*), a pyl-v plant with *aDart* from Nakateaikoku; (*5*), control T-65 *pyl-stb* tester plant. The variegated leaf phenotypes of pyl-v plants with *aDart* from six Guangxi varieties are indistinguishable from each other

Nipponbare on chromosome 6 (Tsugane et al. 2006; M. Maekawa unpublished data), we speculated that the active *aDart* element carried by the seven major cultivars might be either the same *aDart* element in the authentic *pyl-v* line or a closely related element at the same locus on chromosome 6. The hypothesis predicts that the seven varieties containing *aDart* must carry one copy of the *Dart* element at the *iDart1-27* integration site in Nipponbare and that the *aDart* activity must be tightly linked to the *Dart* element in question. As Fig. 2a shows, all eight varieties, including Nakateaikoku, indeed carry a *Dart* element at the anticipated site on chromosome 6. Although these varieties and the T-65 *pyl-stb* tester line are all japonica, we were able to find polymorphism with two SSR markers, RM5509 and RM6811 (McCouch et al. 2002), which are located near the *iDart1-27* locus on chromosome 6 (Fig. 2b), between the varieties and the tester line. The RM5509 and RM6811 genotypes of the pyl-stb segregants in each F2 population showed that *aDart* in all seven varieties examined is tightly linked to the *Dart* element residing at the *iDart1-27* locus; no recombinants bearing both *aDart* and the RM5509 marker from T-65 *pyl-stb* were obtainable, whereas some recombinants with *aDart* and the RM6811 marker from T-65 *pyl-stb* could be detected (Fig. 2b). The results are consistent with the notion that the active *aDart* element in the seven varieties studied resides at the *iDart1-27* locus. Figure 2b also shows that *aDart* in Nakateaikoku is not linked with *Dart* at the *iDart1-27* locus.

Localization of the active *aDart* element in Nakateaikoku

To detect the active *aDart* element in Nakateaikoku, *aDart* was introduced into Kasalath through backcrossing of a leaf-variegated pyl-v F2 segregant of T-65 *pyl-stb* × Nakateaikoku with Kasalath, which carries no active *aDart* element. BC2F2 of pyl-v × Kasalath segregated 279 normal, 32 leaf-variegated pyl-v, and 15 pyl-stb plants. The obtained segregation ratio of pyl-v versus pyl-stb loosely matches the 3:1 ratio ($\chi^2 = 1.20$, $0.25 < P < 0.50$), indicating that a single *aDart* element originated from Nakateaikoku

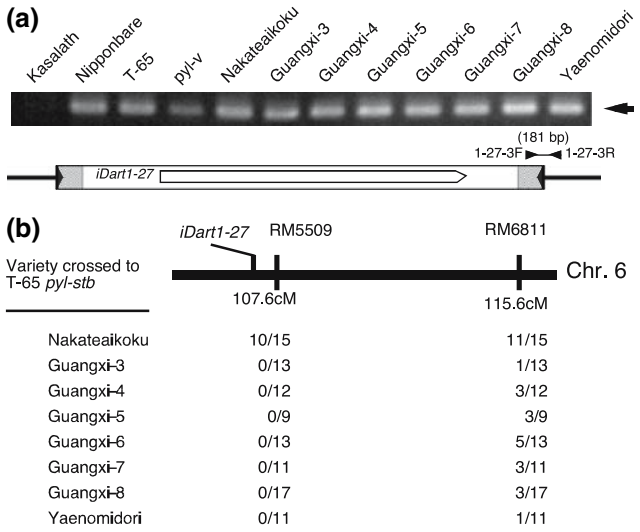

Fig. 2 Characterization of an active *aDart* element in the eight rice varieties. **a** Presence of a *Dart* element at the *iDart1-27* locus on chromosome 6. A *Dart* element at the *iDart1-27* locus was detected by PCR analysis with the primers 1-27-3F and 1-27-3R. The *large horizontal filled arrowheads* at both ends of the *iDart1-27* box indicate the terminal inverted repeats, and the *pentagonal arrow* represents the coding region of the transposase gene. The 181-bp PCR-amplified fragments indicated by the *filled arrow* in PCR analysis were generated from the junction region at the 3′-termini of the *Dart* element. The *small arrowheads* indicate the primers 1-27-3F and 1-27-3R. **b** Linkage between the *Dart* element at the *iDart1-27* locus and an active *aDart* element in the seven varieties. The *iDart1-27* element resides near the SSR markers RM5509 and RM6811 on chromosome 6. The RM5509 and RM6811 genotypes of the pyl-stb segregants in the F2 population from a cross between the *pyl-stb* tester line and a variety containing one copy of an active *aDart* element were scored by PCR analysis

acts on the *pyl-v* allele to induce the leaf-variegated pyl-v phenotype. Thus, 15 pyl-stb plants were selected and used for rough mapping of the *aDart* element from Nakateaikoku with the genome-wide-selected 57 SSR markers described by Maekawa et al. (2005). Because all 15 pyl-stb plants showed the Kasalath-type homozygote of LM5-5 at 49.4 cM of chromosome 5 (Fig. 3a), *aDart* from Nakateaikoku must be located at around 49 cM of chromosome 5. As the Nipponbare genome was reported to contain *iDart1-26* of 3,547 bp at 53 cM of chromosome 5 (Tsugane et al. 2006), we suspected that *aDart* from Nakateaikoku may be a *Dart* element closely related to *iDart1-26* in Nipponbare (Fig. 3b). We first examined whether the Nakateaikoku genome carries a *Dart* element at the *iDart1-26* locus in Nipponbare by PCR analysis. Because PCR amplification of the entire 3,547-bp *iDart1-26* element was found to be difficult, probably due to its high GC content (data not shown), we analyzed the generation of a 211-bp PCR-amplified band derived from the 3′-terminal region of *iDart1-26*. As Fig. 3c shows, Nakateaikoku carries an *iDart1-26*-related element, whereas Kasalath does not. Interestingly, all seven varieties containing *aDart* shown in Table 1 also bear a *Dart*-related element at the *iDart1-26* locus on chromosome 5.

Subsequently, using PCR analysis, we tried to determine whether the *aDart* activity in Nakateaikoku is linked to the presence of the *Dart*-related element at the *iDart1-26* locus. Because Kasalath does not bear the *iDart1-26*-related element, all the plants in the BC2F2 population, which carry *aDart* in either the homozygous or the heterozygous condition and exhibit the leaf-variegated pyl-v phenotype, will produce the 211-bp PCR-amplified band with the primers 1-26-4F and 1-26-4R, which is derived from the 3′-terminal region of the *iDart1-26*-related element, if *aDart* corresponds to the *iDart1-26*-related element. On the other hand, all BC2F2 plants showing the pyl-stb phenotype must generate a 212-bp PCR-amplified band with primers 1-26-1F and 1-26-1R, which is derived from the corresponding locus of Kasalath without the *iDart1-26*-related element. The same 212-bp fragment will also be seen in the pyl-v plants carrying *aDart* in the heterozygous condition. Typical examples of such PCR analyses are shown in Fig. 3d, and the results supported the notion that *aDart* in Nakateaikoku is tightly linked to the *Dart*-related element at the *iDart1-26* locus on chromosome 5; all pyl-v segregants carried the *iDart1-26*-related element, whereas none of the pyl-stb plants contained the *Dart*-related element at the *iDart1-26* locus. PCR analysis of additional 24 pyl-v plants in the BC2F2 population confirmed the results that all pyl-v plants contained the *iDart1-26*-related element (data not shown). A comparison of the results in Fig. 3d further indicated that four pyl-v plants, #1, #3, #6, and #7, bore the *iDart1-26*-related element heterozygously while four other pyl-v plants, #2, #4, #5, and #8, carried the *iDart1-26*-related element homozygously. The segregation patterns of their selfed progeny further confirmed that the former four pyl-v plants bore the active *aDart* elements heterozygously, whereas the latter four pyl-v plants carried *aDart* homozygously. The segregation of the *iDart1-26*-related element coincides with the segregation of the *aDart* activity (Table 2). These results strongly support the hypothesis that *aDart* in Nakateaikoku is the *iDart1-26*-related element on chromosome 5.

Characterization of transcripts produced from *iDart1*-related elements

The *aDart* elements in the seven varieties (six Guangxi lines and Yaenomidori) are likely to be identical to the *iDart1-27*-related element carried by the authentic T-65 *pyl-v* line on chromosome 6, whereas the *aDart* element in Nakateaikoku is likely to be the *iDart1-26*-related element located on chromosome 5. We found that several different mRNA molecules transcribed from *iDart1*-related elements are accumulated in the *pyl-v* line and that these transcripts

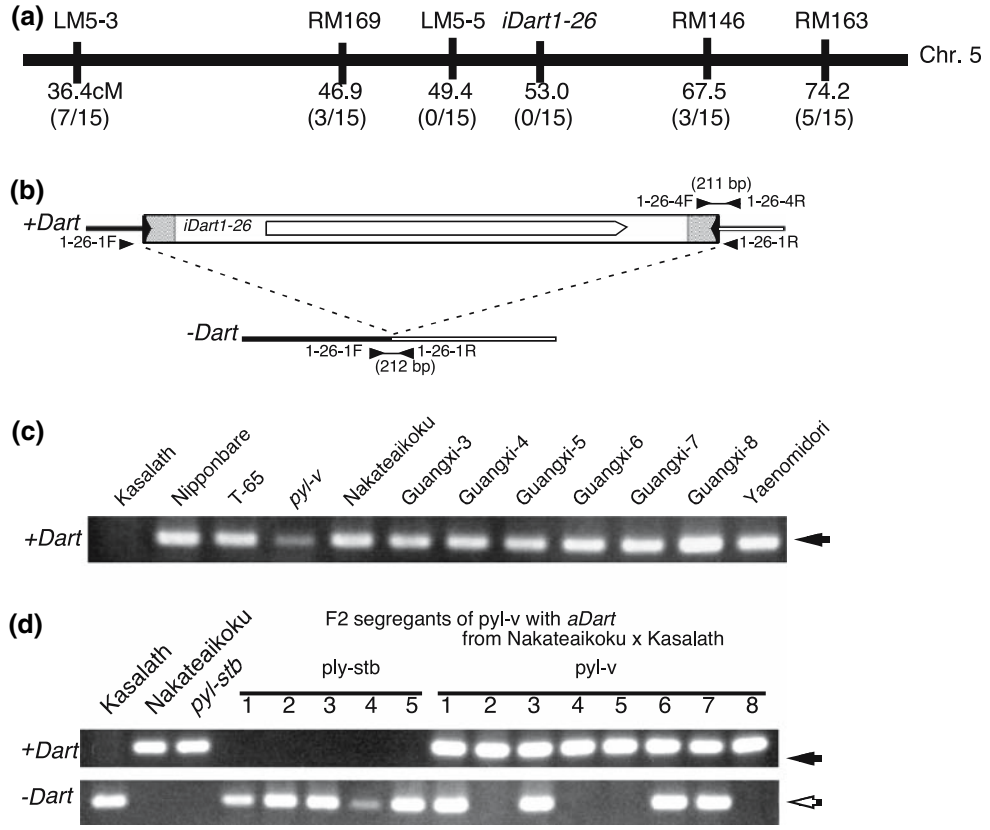

Fig. 3 Characterization of an active *aDart* element in Nakateaikoku. **a** Linkage between the *Dart* element at the *iDart1-26* locus and an active *aDart* element in Nakateaikoku. The *iDart1-26* element resides on chromosome 5. The genotypes of the SSR markers on chromosome 5 among the 15 pyl-stb segregants in the F2 population from a cross between a pyl-v segregant (obtained from the F2 population from a cross between Nakateaikoku and the *pyl-stb* tester line) and Kasalath, which shows no *aDart* activity, were scored by PCR analysis. **b** Genomic structures at the *iDart1-26* locus with and without *Dart*. The symbols are as in Fig. 2, and the 211-bp PCR-amplified fragments with the primers 1-26-4F and 1-26-4R were generated from the junction region at the 3′-termini of the *Dart* element. In the absence of *Dart*, the 212-bp fragments were produced in PCR analysis with the primers 1-26-1F and 1-26-1R. **c** Presence of a *Dart* element at the *iDart1-26* locus on chromosome 5. A *Dart* element at the *iDart1-26* locus was detected by the production of the 211-bp fragment pointed by the *filled arrow* in PCR analysis with the primers 1-26-4F and 1-26-4R. **d** Cosegregation of the *iDart-26*-related element and leaf variegation in the F2 population from a cross between the pyl-v segregant and Kasalath. Only variegated pyl-v plants carry the *iDart-26*-related element, whereas all pyl-stb plants and some pyl-v plants exhibit PCR-amplified fragments derived from the Kasalath genome, which contains no *iDart-26*-related element. The *filled* and *open arrows* indicate the 211-bp and 212-bp PCR-amplified fragments with the primers 1-26-4F and 1-26-4R and the primers 1-26-1F and 1-26-1R, respectively. The presence and absence of the *iDart-26*-related element are indicated by *+Dart* and *−Dart*, respectively

Table 2 Segregation of pyl-v and ply-stb plants in the selfed progeny of a *pyl-v* plant carrying an active *aDart* element(s) from Nakateaikoku

pyl-v	Selfed progeny		Total	pyl-stb (%)
	pyl-v	pyl-stb		
#1	21	5	26	19.2
#2	36		36	
#3	54	16	70	22.9
#4	38		38	
#5	32		32	
#6	41	12	53	22.6
#7	24	5	29	17.2
#8	21		21	

comprise 5′-untranslated exon 1 and exon 2 that contains the entire coding region of the *Dart1* transposase (K. Tsugane and S. Iida, unpublished). We hypothesized that the transcripts from *iDart1-27*- and *iDart1-26*-related elements must be accumulated in the major seven varieties and in Nakateaikoku, respectively. Because polymorphism can be found within the 737-bp 5′-terminal segment consisting of 54-bp 5′-UTR and 683-bp 5′ coding sequence in the expressed *iDart1* transposase transcripts (Fig. 4a), sequencing of the 737-bp segments allow us to identify which *iDart1*-related elements produce the mRNAs of the *iDart1* transoposase gene. RT-PCR analysis with primers Dart-F1 and Dart-R1 revealed that the expected transcripts were present in all eight varieties examined (Fig. 4b). The

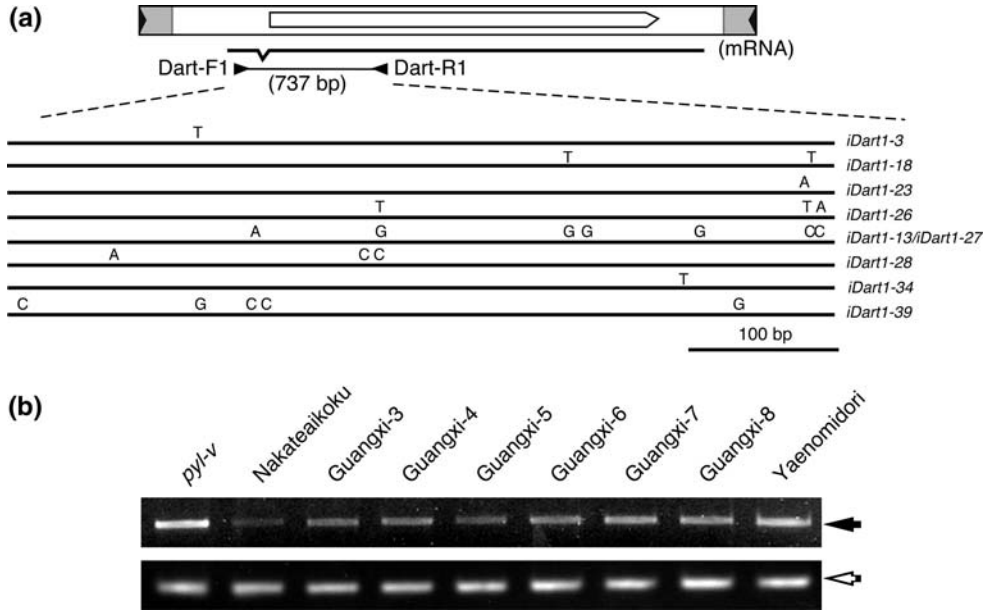

Fig. 4 Characterization of the transcripts from *iDart1*-related elements in the eight rice varieties. **a** Structure of the transcripts from *iDart1*-related elements and schematic presentation of polymorphism found in the 5′ region of the transcripts. The gene for the *iDart1* transposase carries an about 70-bp intron in its 5′-untranslated region, and the 737-bp segments to be sequenced were obtained by RT-PCR-amplification with primers Dart-F1 and Dart-R1. Only single-base alterations in the 737-bp region were shown. The other symbols are as in Fig. 2. Note that the transcripts from *iDart1-13*- and *iDart1-27*-related elements could not be distinguished by their sequences. **b** Presence of the transcripts from *iDart1*-related elements. The 737-bp segments from the transcripts from *iDart1*-related elements were pointed by the *filled arrow* in RT-PCR analysis with primers Dart-F1 and Dart-R1. The *open arrow* indicates the mixture of the 438-bp segments derived from transcripts of the four constitutively expressed genes encoding elongation factor 1α proteins, used as an internal control

transcripts in Nakateaikoku appeared to be less accumulated than those in other seven varieties as well as the authentic *pyl-v* line, providing an explanation that Nakateaikoku displays lighter leaf-variegation than the major seven lines (Fig. 1). Although we were unable to distinguish the *iDart1-13* transcripts from the *iDart1-27* transcripts, it is clear that major mRNAs found in Nakateaikoku are transcribed from *iDart1-26* while those in other seven varieties are from *iDart1-13/iDart1-27* (Table 3). The results support the notion that the active *aDart* elements in the major seven varieties and Nakateaikoku are *iDart1-27*- and *iDart1-26*-related elements, respectively.

Distribution and excision of endogenous *nDart1* elements in the eight varieties carrying *aDart*

Nipponbare carries 13 *nDart1* elements, and the authentic mutable line T-65 *pyl-v* contains at least 12 *nDart1*

Table 3 Occurrence and distribution of the transcripts from *iDart1*-related elements detected in eight rice varieties carrying an active *aDart* element

Variety analyzed	Clones sequenced	*iDart-1-3*	*iDart1–18*	*iDart1–23*	*iDart1–26*	*iDart1–13* / *iDart1–27*[a]	*iDart1–28*	*iDart1–34*	*iDart1–39*	Others[b]
Nakateaikoku	36	2 (6)		3 (8)	26 (72)					5 (14)
Guangxi-3	12					12 (100)				
Guangxi-4	10					10 (100)				
Guangxi-5	19					17 (89)	1 (5)	1 (5)		
Guangxi-6	11					9 (82)	2 (18)			
Guangxi-7	17		1(6)			14 (82)	2 (12)			
Guangxi-8	17	1 (6)				15 (88)	1 (6)			
Yaenomidori	13	1 (8)				10 (77)			1 (8)	

The transcripts from *iDart1-27*- and *iDart1-26*-related elements were identified by determining the sequence of the individual RT-PCR-amplified product cloned. Distribution of the transcripts from different *iDart1*-related element (%) is shown in parentheses

[a] See Fig. 4a

[b] The sequences of the transcripts did not match those of the anticipated transcripts from *iDart1*-related elements in Nipponbare

elements at the same loci to Nipponbare in addition to *nDart1-0* within the *OsClpP5* gene (Tsugane et al. 2006). Among the 12 endogenous *nDart1* elements in the T-65 *pyl-v* line, the excision frequencies of two elements, *nDart1-3*(*3-1*) and *nDart1-12*, on chromosomes 3 and 1, respectively, were much higher than those of the other *nDart1* elements. We first examined whether these eight varieties carry the endogenous *nDart1* elements at the loci common to Nipponbare and whether some of those present in their genomes are somatically excised (Table 4). Six Chinese varieties, Guangxi-3 to -8, and Yaenomidori carry only six *nDart1* elements common to Nipponbare, and none of these *nDart1* elements were shown to be excised even though they contain *aDart*. We also noticed that *nDart1-1* in these varieties, as well as *nDart1-2* in Yaenomidori, was difficult to detect by PCR analysis, probably because the primer(s) used could not anneal properly with the genomic target sequence(s). Nakateaikoku bears nine *nDart1* elements, including *nDart1-3*(*3-1*) and *nDart1-12*, and the excision frequencies of these two elements are lower than those of the corresponding *nDart1* elements in T-65 *pyl-v* (Table 4). Thus, the excision of *nDart1-0* at the mutable *pyl-v* locus (Fig. 1), as well as those of the endogenous *nDart1-3*(*3-1*) and *nDart1-12* elements in Nakateaikoku (Table 4), appears to be coordinately lower than those in the authentic T-65 *pyl-v* mutable line.

Discussion

Although several mutable alleles caused by endogenous DNA transposons, including *mPing*, *nDart*, and *dTok* in rice, have been documented (Nakazaki et al. 2003; Fujino et al. 2005; Tsugane et al. 2006; Moon et al. 2006), the *nDart/aDart* system currently occupies a unique position among these rice DNA transposon systems because (1) several mutable alleles caused by the insertion of *nDart1* have already been identified (Fujino et al. 2005; Tsugane et al. 2006; M. Maekawa, K. Takagi, K. Tsugane, unpublished data), (2) the transposition of *nDart1* has been shown to be controlled by ordinary crossing under natural growth conditions (Maekawa et al. 1999; Tsugane et al. 2006), and (3) *nDart1* tends to integrate into the exon or 5′-UTR regions with relatively higher GC-rich sequences (Takagi et al. 2007; K. Takagi, M. Maekawa, unpublished data). These features also provide certain advantages over the currently available gene-tagging systems in rice, including the utilization of foreign elements, such as T-DNA or maize DNA transposons *Ac/Ds* and *En/Spm* and the endogenous retrotransposon *Tos17*, all of which require tissue cultures to either introduce these foreign elements into rice calli or to activate dormant *Tos17* in the genome (Hirochika et al. 2004; Guiderdoni et al. 2007; Zhu et al. 2007); this is because no somaclonal variation due to tissue cultures (Larkin and Scowcroft 1981) is expected to occur in our *nDart/aDart* system. As a large-scale collection of *nDart1* insertional mutants can be established at low cost under normal growth conditions (M. Maekawa, unpublished data), we searched for the distribution of an active *aDart* element that can mobilize *nDart1-0* inserted in the *OsClpP5* gene among 100 rice cultivars, including 51 indica varieties (Suppl. Table 1), by crossing with the T-65 *pyl-stb* tester line for the very efficient detection of an active *aDart* element (Tsugane et al. 2006). Only eight japonica varieties, two from Japan and six from China, were found to carry *aDart* (Fig. 1; Table 1). Judging from the distribution patterns of the *nDart1* elements (Table 4), all six Chinese varieties are very similar to each other and also relatively similar to Yaenomidori, but they are distantly related to Nipponbare and T-65. Interestingly, Nakateaikoku contains both *nDart1-3*(*3-1*) and *nDart1-12*, whose excisions occur at higher rates than those of other *nDart1* elements in Nakateaikoku as well as in the authentic T-65 *pyl-v* line, whereas the six Chinese varieties do not bear these two *nDart1* elements.

Based upon the leaf variegation phenotypes in the pyl-v segregants carrying active *aDart* elements, eight cultivars can be classified into two groups, a major group consisting of one Japanese and six Chinese varieties exhibiting variegated leaves as heavy as the authentic T-65 *pyl-v* line, and a minor group formed by a single Japanese cultivar, Nakateaikoku, displaying lighter leaf variegation than the varieties in the major group (Fig. 1). All active *aDart* elements in the major group were shown to be tightly linked to the *Dart*-related element corresponding to *iDart1-27* on chromosome 6 in Nipponbare (Fig. 2). As the active *aDart* element in the authentic T-65 *pyl-v* line has been identified to coincide with the *iDart1-27* element by a fine-mapping method (Tsugane et al. 2006; M. Maekawa, Z. Shimatani, K. Takagi, K. Tsugane, S. Iida, unpublished data), it is highly likely that the *aDart* elements in the major group also correspond to the *iDart1-27*-related element. Subsequently, we were able to map the *aDart* element in the minor group, Nakateaikoku, to be tightly linked to the *Dart*-related element corresponding to *iDart1-26* in Nipponbare (Fig. 3). Moreover, the transposase gene in the *iDart1-27*-related element was found to be predominantly expressed in the major group, whereas that in the *iDart1-26*-related element was mainly expressed in Nakateaikoku (Fig. 4). The sequences of *iDart1-27*-related elements in the major group as well as that of *iDart1-26*-related element in Nakateaikoku appear to be conserved, because their sequenced regions were found to be identical to the corresponding sequences of the *iDart1* elements in Nipponbare. Although we were able to map the active *aDart* element in the major group, our data cannot exclude a remote possibility

Table 4 Excision of nonautonomous elements *nDart1-0* and its relatives in eight rice varieties carrying an active *aDart* element

nDart1-3 subgroup	Chr.	Position (Mbp[a])	Nipponbare	T-65	T-65 pyl-v (*aDart1-27*)	Nakateaikoku (*aDart*)	Guangxi-3 (*aDart*)	Guangxi-4 (*aDart*)	Guangxi-5 (*aDart*)	Guangxi-6 (*aDart*)	Guangxi-7 (*aDart*)	Guangxi-8 (*aDart*)	Yaenomidori (*aDart*)
nDart1-0	3	10.9	Abs	–	+++	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs
nDart1-1	9	13.7	–	–	–	–	Ndt	Ndt	Ndt	Ndt	Ndt	Ndt	Ndt
nDart1-2	12	6.6	–	–	–	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Ndt
nDart1-3(8)	8	6.3	–	–	–	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs
nDart1-3(6)	6	30.0	–	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs
nDart1-3(3-1)	3	2.6	–	–	++	+	Abs	Abs	Abs	Abs	Abs	Abs	Abs
nDart1-3(3-2)	3	30.0	–	–	–	–	–	–	–	–	–	–	–
nDart1-5	3	6.3	–	–	–	–	Abs	Abs	Abs	Abs	Abs	Abs	Abs
nDart1-6	2	34.2	–	–	–	–	–	–	–	–	–	–	–
nDart1-7	4	18.3	–	–	–	–	–	–	–	–	–	–	–
nDart1-9	2	16.4	–	–	–	–	–	–	–	–	–	–	–
nDart1-10	5	9.1	–	–	–	–	–	–	–	–	–	–	–
nDart1-11	3	20.1	–	–	–	–	–	–	–	–	–	–	Ndt
nDart1-12	1	39.1	–	–	++	+	Abs	Abs	Abs	Abs	Abs	Abs	–

The symbols +++, ++, + and −indicate the *nDart1* elements present in each genome and excised with high, moderate, scarce, and undetectable frequencies, respectively. Abs means that the *nDart1* element in question is absent by PCR analysis, whereas Ndt indicates that the presence or absence of *nDart1* could not be determined because no PCR-amplified bands were detectable

[a] The positions of the nonautonomous *nDart1* elements are based on pseudomolecules var. 4.0

that two copies of *iDart1-27*-related *aDart* element reside adjacent to each other on chromosome 6. Judging from both the leaf variegation patterns (Fig. 1) and the amounts of the transposase transcripts accumulated (Fig. 4), the activity of *aDart* in the major group appears to be very similar to each other and stronger than that in Nakateaikoku. It is well known that the maize element *Ac*, a representative element in the *hAT* superfamily, often displays dosage dependent variegation patterns (Kunze and Weil 2002). The leaf-variegation of the authentic T-65 *pyl-v* line (Fig. 1) was shown to be promoted by one copy of *aDart* belonging to the *hAT* superfamily (M. Maekawa, Z. Shimatani, K. Takagi, K. Tsugane, S. Iida, unpublished data). Because the leaves of the seven major varieties and the *pyl-v* line, all of which are promoted by the *iDart1-27*-related *aDart* element, exhibit very similar variegation patterns (Fig. 1), we favor the idea that all of them carry only one copy of active *aDart* element on chromosome 6.

While the indica variety Kasalath contains neither an *iDart1-27*-related nor an *iDart1-26*-related element, all eight cultivars, as well as the Nipponbare, T-65, T-65 *pyl-v*, and T-65 *pyl-stb* lines, carry both elements (Fig. 2). Yet, the major seven cultivars appear to carry an active *aDart* element at the *iDart1-27* locus on chromosome 6, as is the case for the authentic T-65 *pyl-v* line. Nakateaikoku is likely to bear *aDart* at the *iDart1-26* locus on chromosome 5 while Nipponbare, T-65, and the T-65 *pyl-stb* tester line contain no active *aDart* element at all. Nipponbare contains about 35 copies of *iDart1*-related elements, which carry a potentially active but epigenetically silenced transposase gene, and 5-azacytidine treatments of Nipponbare seeds activated the dormant *iDart1*-related elements and induced the excision of certain *nDart1* elements (Tsugane et al. 2006; Takagi et al. 2007; C.-H. Eun, M. Maekawa, unpublished data). We do not know whether only a portion of dormant *iDart1*-related elements in Nipponbare can be activated by the 5-azacytidine treatments. Neither do we know why only the *Dart*-related element corresponding to *iDart1-27* and *iDart1-26* in the major seven varieties and Nakateaikoku, respectively, has escaped from epigenetic gene silencing. In this respect, it should be emphasized that no apparent breeding efforts have been made to retain the *aDart* activity in the cultivated rice varieties described here, probably because highly frequent occurrence of mutable alleles often conferring certain deficiencies in established breeding lines may be disadvantageous. By contrast, considerable efforts have often been devoted to maintain characteristic traits for many flower variegations caused by mutable alleles in the presence of corresponding active autonomous elements, because certain aesthetic values are found in not only variegated phenotypes but also frequent generation of new varieties showing floriculturally important traits (Iida et al. 2004; Chopra et al. 2006). Nonetheless, the identification of *aDart* and its distribution among 100 cultivars described here would shed light on an evolutionary aspect of the *nDart/aDart* system in rice. The localization of the active *aDart* element would certainly facilitate an efficient and somaclonal variation-free gene tagging system promoted by the *nDart/aDart* elements in japonica rice. Moreover, the introduction of both the mutable *pyl-v* allele composed by the insertion of *nDart1-0* into *OsClpP5* and the active *aDart* element into indica varieties would enhance the possibility of developing a new inexpensive and somaclonal variation-free gene tagging system in various indica cultivars grown under ordinary conditions.

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