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ORIGINAL PAPER

Multiple alleles at *Early flowering 1* locus making variation in the basic vegetative growth period in rice (*Oryza sativa* L.)

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Abstract A recently established rice breeding program in low latitudes aims to develop varieties with extremely long basic vegetative growth (BVG) periods and weak photoperiod sensitivities. The Taiwanese *japonica* variety Taichung 65 (T65) harbors a recessive allele *ef1* at the *Ef1* (*Early flowering 1*) locus, thereby exhibiting an extremely long BVG period. The previous reported functional allele *Ehd1* (*Early heading date 1*), located on chromosome 10, encodes a B-type response regulator, thereby shortening the BVG period, whereas its nonfunctional allele *ehd1* greatly prolongs the BVG period. A conventional analysis using F_2 and F_3 populations and a subsequent CAPS analysis based on the amino acid sequences of *Ehd1* and *ehd1* showed that *Ef1* and *Ehd1* were at the same locus. The CAPS analysis also indicated that the Taiwanese *japonica* varieties with extremely long BVG periods all harbor *ef1*, but that *ef1* does not exist among *indica* and *japonica* varieties in the low latitudes. Since *ef1* has not been found in any *japonica* varieties outside Taiwan, this allele might have originated in Taiwan. Sequence analysis revealed that the mutant allele *ef1-h*, which prolongs the BVG period even more than *ef1* does, harbors an *mPing* insertion in exon 2, which causes the complete loss of gene function. Our results indicate that both *ef1* or *ef1-h* alleles can be used as new gene sources in developing rice varieties with extremely long BVG periods for low latitudes.

Abbreviations

BVG	Basic vegetative growth
CAPS	Cleaved amplified polymorphic sequence
BVP	Basic vegetative growth phase
PSP	Photoperiod sensitive phase
RP	Reproductive phase
GARP	*Golden2, Arabidopsis RESPONSE REGULATOR* (ARR), and *Chlamydomonas* regulator protein of P-starvation acclimatization response (*Psr1*)

Introduction

Flowering time (heading time) plays a principal role in the regional adaptability of rice (Okumoto et al. 1991, 1992a, b, 1996; Tanisaka et al. 1992; Ichitani et al. 1997, 1998, 2002). Preflowering development of rice is divided into three successive phases: basic vegetative growth phase (BVP), photoperiod-sensitive phase (PSP), and reproductive phase (RP) (Vergara and Chang 1985; Yin et al. 1997; Nishida et al. 2001). Different varieties of rice exhibit great variation in the durations of the first two phases, while the duration of the second is almost constant at about 35 days, regardless of rice variety or photoperiod (Vergara and Chang 1985). Because PSP can be completely eliminated

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under optimum photoperiod conditions, heading time of rice has been considered to be primarily determined by the duration of the basic vegetative growth period (BVG period, nearly equal to the duration of BVP plus that of RP) under optimum photoperiod conditions, expressed in days to heading (Hosoi 1981; Tanisaka et al. 1992), and by the plant's photoperiod sensitivity (PS).

A recently established rice breeding program serving low latitudes aims to develop varieties with extremely long BVG periods and weak PS. Such varieties exhibit almost constant vegetative growth periods in order to achieve sufficient vegetative growth in low latitudes, where short photoperiod conditions continue almost throughout the year, to permit stable and high-yielding production, as well as double or triple cropping. It is therefore important to understand the genetic factors involved in determining both the BVG period and the PS of rice germplasm. Unfortunately, far less progress has been made toward a complete genetic and physiological analysis of the BVG period than that of PS. This is largely due to the difficulty of genetic analysis of the BVG period, which requires short-photoperiodic growing conditions that suppress the segregation noises of PS loci.

To date, seven loci involved in determining the BVG period have been reported: *Ef1* on chromosome 10 (Tsai 1986), *Ef2* on chromosome 9 (Khun et al. 2005b), *Ef3* (chromosome unknown; Tsai 1991), *Ef4* on chromosome 3 (Khun et al. 2005a), *Ef5* on chromosome 6 (Khun et al. 2004), *Ef6* on chromosome 7 (Khun et al. 2006), and *Ehd1* on chromosome 10 (Doi et al. 1998). Among them, *Ef1* was the first BVG locus identified: the dominant allele *Ef1* confers a short BVG period, while the recessive allele *ef1* confers an extremely long BVG period. This locus has been extensively investigated in several studies aiming to identify the allele that is present in certain *japonica* varieties and to observe the gene's effects on preflowering developmental phases and its interactive effects with other loci (Sato and Hayashi 1985; Inoue et al. 1998; Nishida et al. 2001; Ichitani et al. 2002). These studies have shown that *Ef1* is a key locus dividing *japonica* rice varieties into two groups: an *ef1* group found in the semi-low latitudes (21–26°N), and an *Ef1* group found in temperate zones (25–39°N) (Sakamoto and Toriyama 1967; Sato and Hayashi 1985). The distribution of alleles at the *Ef1* locus among *indica* and *japonica* varieties cultivated in low latitudes (lower than 26°N or S) has not yet been investigated. In addition to *ef1*, the *Ef1* locus has another recessive allele, *ef1-h*, which was induced by gamma irradiation of seeds of the Japanese *japonica* variety Gimbozu (Nishida et al. 2002). This mutant allele has a greater elongating effect on the BVG period (Nishida et al. 2002) than *ef1*, adding 45.0 more days to the BVG period than *ef1* does, whereas *ef1* permits a residual function to accelerate heading. The reason for the two alleles' different elongating effects on BVG has not yet been elucidated. Thus, *Ef1* locus has been extensively investigated from various view points, but its physical map position, as well as its nucleotide sequence, has not yet been identified.

The *Ehd1* locus was originally identified as a heading time QTL (quantitative trait locus) on chromosome 10 in an experiment using the cross combination between T65 and an accession of African rice (*Oryza glaberrima* Steud.; Acc. IRGC 104038) (Doi et al. 1998): a dominant allele *Ehd1-gla* from the African rice confers a short BVG period, while the recessive allele *ehd1*, found in T65, confers an extremely long BVG period. Later, Doi et al. (2004) successfully isolated *Ehd1* and *ehd1*, and showed that the former encoded a B-type response regulator accelerating flowering, while the latter resulted in loss of gene function. As described above, *Ef1* locus is located near *Ehd1* locus on chromosome 10, and its effect on the BVG period is similar to that of *Ehd1* locus. This suggests that *Ef1* locus is identical to *Ehd1* locus, but their allelic relationship has not yet been shown. If *Ehd1* locus proves to be identical with *Ef1* locus, we will be able to accumulate and integrate a lot of information both from studies on *Ef1* locus and from those on *Ehd1* locus, which have been conducted independent of each other.

In the present study, we first sought to discover whether the *Ef1* locus is identical to the *Ehd1* locus, using a segregation analysis of heading time under both natural and short photoperiod conditions. To confirm the result, we conducted a cleaved amplified polymorphic sequence (CAPS) analysis peculiar to the *Ehd1* locus based on the results of Doi et al. (2004). They demonstrated that *Ehd1* and *ehd1* can easily be discriminated from each other by the number of the *Dde*I restriction sites in the GARP [*Golden2, Arabidopsis RESPONSE REGULATOR* (ARR), and *Chlamydomonas* regulator protein of P-starvation acclimatization response (*Psr1*)] DNA-binding domain in the middle of them: *Ehd1* harbors a single *Dde*I restriction site in the GARP DNA-binding domain, whereas *ehd1* harbors two restriction sites, one of which was caused by the amino acid substitution from Gly to Arg due to a single nucleotide substitution from G to A. Since restriction site(s) is peculiar to the *Ehd1* alleles, we can easily investigate the difference between *Ehd1* alleles and *Ef1* alleles; therefore, we used the CAPS analysis instead of sequence analysis. Subsequently, since the *Ef1* and the *Ehd1* were found to be the same locus, we examined the distribution of the recessive allele *ef1* among *indica* varieties using the CAPS analysis. We also examined differences in the nucleotide sequences of *ef1* and *ef1-h*. Based on the results of these experiments, we discussed the roles of the *Ef1* locus in low-latitude rice production.

Estimates of the duration of BVP require either photoperiod transfer treatment (Ellis et al. 1992) or microscopic

observation of the reproductive shoot apex, but both procedures are difficult to perform on a large number of plants. Instead, previous genetic studies on the duration of BVP all have used the BVG period including the days to heading (DH) under short photoperiod conditions. Furthermore, all known BVP loci were identified by analyzing the BVG period. Thus, 'the BVP loci' identified so far should be called 'the BVG loci'. In the present study, we conducted genetic analyses of the duration of the BVG period instead of the duration of BVP. For this reason, in this paper we avoided to use the term 'BVP'.

Materials and methods

Allelism test between *Ef1* and *Ehd1-gla* (Experiment 1)

Two F_2 populations from T65e/T65 (284 plants) and T65e/T65Eb (269 plants) crosses were grown under natural photoperiod conditions in a paddy field in Kyoto (35°01′N), Japan. None of the *Ef1* alleles have been sequenced, and few DNA markers are available in these two cross-combinations because these lines were near-isogenic to each other; hence, we conducted segregation analysis for heading time. T65 is the Taiwanese *japonica* variety developed in 1936, and T65Ea and T65Eb are near-isogenic lines of T65 for the *Ef1* locus: T65 carries the nonfunctional allele *ef1* at the *Ef1* locus, while T65Ea and T65Eb carry functional alleles, *Ef1-a* and *Ef1-b*, respectively. The *Ef1-a* allele was introduced to T65 from the Chinese *japonica* variety Tatung-tsailai, while the *Ef1-b* allele was introduced from the Japanese *japonica* variety Bozu 5 (Tsai 1986). T65e is a near-isogenic line of T65 for the *Ehd1* locus, carrying the functional allele *Ehd1-gla*, which was introduced from an accession of *O. glaberrima* (Doi et al. 2004). Seeds were sown in a nursery bed on May 15, 2005, and transplanted into the paddy field 30 days later. Plant spacing was 10 × 30 cm, and N, K_2O and P_2O_5 fertilizers were applied in quantities of 6, 9 and 9 kg/100 m^2, respectively.

The progeny test was also conducted in 2006 with 152 F_3 lines for the T65e/T65 cross and 149 F_3 lines for the T65e/T65Eb cross. In the progeny test, the F_3 lines were grown in pots under a 10 h photoperiod (short photoperiod). Each F_3 line was the progeny of an F_2 plant randomly selected from among all F_2 plants. Twenty-five germinated seeds per line were sown in a 30 × 60 cm tray filled with field soil and covered with granulated soil. Plant spacing was 5 × 5 cm, and a 1-L HYPONeX (mixed fertilizer including 6% nitrogen, 10% water-soluble phosphoric acid and 5% water-soluble potassium; HYPONeX Co. Ltd, Osaka, Japan) solution was applied as an additional fertilizer at 1.3% (w/v) per tray every other week. Heading time was recorded for each individual F_2 and F_3 plants when the first panicle emerged from the leaf sheath of the flag leaf.

Genetic analysis of BVG periods on selected Taiwanese *japonica* varieties (Experiment 2)

T65 and four other Taiwanese *japonica* varieties, Tainang 5 (TN5), Tainang 7 (TN7), Tainang 9 (TN9) and Tainung 70 (TU70), were grown under a 10 h photoperiod (short photoperiod) so that we could estimate their BVG period. Seeds were disinfected with Benlate solution (Sumitomo Chemical Co. Ltd, Tokyo, Japan), diluted 1,000-fold with water, at 20°C for 24 h, and pre-germinated at 30°C for 2 days. Ten seeds were sown in each 3.6-L pot filled with field soil, and covered with granulated soil. Five such pots were sown for each variety. Seedlings were thinned to five plants per pot 14 days after sowing. The BVG period of each line was indicated by the average number of days to heading of two pots. Since each of these four varieties exhibited a BVG period almost identical to that of T65, a genetic analysis of their long BVG periods was conducted using eight F_2 populations from crosses of each of the four varieties with T65 and T65Eb. The F_2 populations were grown in trays under a 10 h photoperiod. Germinated seeds were sown in a tray filled with field soil and covered with granulated soil. Other experimental procedures were similar to those described for the F_3 progeny test in Experiment 1.

Since segregation analysis for *Ef1* locus clearly showed that all four varieties carry *ef1* allele at the locus, the long BVG periods of these varieties are attributable to the presence of *ef1* allele at *Ef1* locus. Therefore, we attempted to ascertain if they, too, carried *ehd1* at the *Ehd1* locus by performing a CAPS analysis. According to Doi et al. (2004), the functional allele *Ehd1-Nip* of the *japonica* variety Nipponbare, which is isoallelic to *Ehd1-gla*, harbors one *Dde*I site in its GARP DNA-binding domain and produces two fragments (137 and 798 bp) with *Dde*I digestion. The nonfunctional allele *ehd1* (=*ef1*) of T65, in contrast, harbors two *Dde*I sites due to a Gly to Arg substitution in the GARP DNA-binding domain, which is thought to be crucial for the null function of *ef1*, and produces three fragments (137, 216 and 582 bp) with *Dde*I digestion. A pair of PCR primers (Ehd1_U5: 5′-CCGGTCATCCTCCATCAATA-3′, Ehd1_L5: 5′-CCATCGGTTCTATAAAAA-3′) were designed to amplify the 935 bp segment. PCR was carried out with 10 µl reaction mixture containing 1 µl template DNA, 10× PCR buffer, 25 mM $MgCl_2$, 2 mM of each dNTP, 0.1 µl *Taq* DNA polymerase (5U/µl), 2 µl of a 2.5 mM solution of each primer, and 1.6 µl H_2O. PCR conditions were as follows: 94°C for 5 min, followed by 35 cycles (1 min at 94°C, 1 min at 55°C, and 2 min at 72°C) with a final extension of 7 min at 72°C. The amplified products were digested with *Dde*I at 37°C for 1 h. Amplicons

and digested amplicons were separated on 1% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and the DNA fragments were visualized under UV light.

Distribution of *ehd1* (=*ef1*) alleles among Asian rice varieties (Experiment 3)

An additional CAPS analysis was conducted to investigate the distribution of *ehd1* (=*ef1*) on 90 selected varieties, which include 50 Taiwanese varieties, 7 Indonesian varieties, 10 Bangladesh varieties, 21 Chinese *japonica* varieties and 2 IRRI (International Rice Research Institute) varieties. The list of names is given in Supplemental table.

Sequence analysis of *ef1-h* gene (Experiment 4)

The results of Experiments 1 and 3 had shown that *Ef1* and *Ehd1* were the same locus, and that *ef1* was identical to *ehd1*. Next, we attempted to compare the sequence of the mutant allele *ef1-h* with that of *ehd1* (=*ef1*). This mutant allele was identified in a late heading time mutant line HS169 (Nishida et al. 2002), which was induced with gamma irradiation of seeds of the *japonica* variety Gimbozu (Tanisaka et al. 1992). Nishida et al. (2002) reported that *ef1-h* conferred an extremely long BVG period, and that the effect of *ef1-h* was much greater than the complementary effect of *ehd1* (=*ef1*) and a recessive allele of the *Se1* (*photoperiod sensitivity 1*) locus. It is therefore considered that *ehd1* (=*ef1*) permits some residual function accelerating heading, while *ef1-h* loses the function entirely. PCR was conducted with a primer pair designed from the sequence of an *Ehd1* allele of the *japonica* variety Nipponbare (*Ehd1-Nip*, AB092506). Total genomic DNA was extracted from fresh leaves (ca. 100–200 mg). PCR products were separated by electrophoresis on a 2% agarose gel and purified with a Qiaquick Gel Extraction kit (Qiagen Sciences, Germantown, MD, USA). The purified DNA fragments were sequenced using a Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit and a CEQ8000 (Beckman Coulter Inc., Fullerton, CA, USA).

Results

Allelism test between *Ef1* and *Ehd-gla* (Experiment 1)

The F_2 population of the T65e (*Ehd1-glaEhd1-gla*)/T65 (*ef1ef1*) cross showed a bimodal distribution within the parental ranges, with a clear break point dividing the population into early (T65e type) and late (T65 type) heading time groups (Fig. 1a). The ratio of early type: late type fit the 3:1 ratio expected for one-locus segregation ($\chi^2 = 0.920$, $P = 0.337$). A progeny test showed that all 152 F_3 lines could be classified into three groups. The ratio of 35:71:46 for [T65e type]:[segregating type]:[T65 type] lines fit the 1:2:1 ratio expected for one-locus segregation ($\chi^2 = 2.250$, $P = 0.324$) (Table 1). This indicates that *Ehd1-gla* is allelic to *ef1*.

The F_2 population of the T65e (*Ehd-glaEhd1-gla)*/T65Eb (*Ef1-bEf1-b*) cross showed a narrow range of distribution without any transgressive segregants (Fig. 1b), and no segregating F_3 lines appeared (data not shown). This indicates that both *Ehd1-gla* and *Ef1-b* are alleles with similar functions at the *Ef1* locus, although it remains unknown whether these two alleles are identical or isoallelic to each other. According to Doi et al. (2004), *Ehd1-gla* and *ehd1* are allelic to one another, and *ehd1* was identified in T65; therefore we assumed that *ehd1* is identical to *ef1*. Hereafter, we use the gene symbols *Ef1* and *ef1* for *Ehd1* and *ehd1*, respectively.

Genetic analysis of BVG periods on selected Taiwanese *japonica* varieties (Experiment 2)

The four Taiwanese *japonica* varieties, TN5, TN7, TN9, and TU70, all exhibited 22.6–36.2 days longer BVG periods than T65Eb (47.7 days) (Table 2), although there

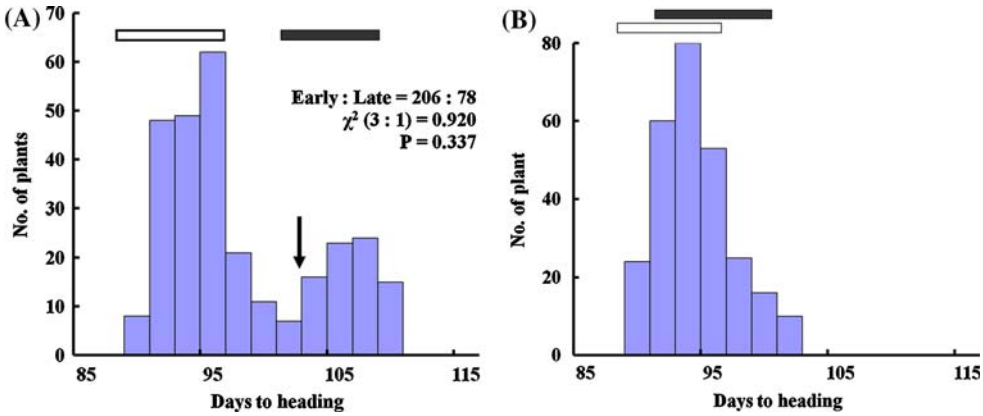

Fig. 1 Frequency distributions of days to heading in two F_2 populations, one from the cross of T65e × T65 (**a**) and one from the cross of T65e × T65Eb (**b**), under natural photoperiod conditions. A *white box* and a *black box* indicate the parental ranges of days to heading. An *arrow* indicates a breakpoint between early heading type and late heading type

Table 1 Frequency distributions of days to heading in representive F_3 lines (T65 × T65e)

Parents and F_3 lines[a]	Genotype of parent and F_2 plants[b]	Days to heading[c]														Total	No. of F_3 lines observed[d]
		77	79	81	83	85	87	89	91	93	95	97	99	101	103		
T65	*ehd1ehd1*										4	6	9	2	1	22	
T65e	*Ehd1Ehd1*		1	2	8	9	1									21	
TF-2	*Ehd1Ehd1*		7	12	(4)											23	35
TF-3			5	(14)	6											25	
TF-15			2	17	4	(1)										24	
TF-21		1	6	9	8	◯										24	
TF-3	*Ehd1ehd1*		3	12	(2)	1					1	2	3	1		25	71
TF-7			4	7	(5)	2				1	2	3			1	25	
TF-18			4	8	4	(1)					4	3		1		25	
TF-20			2	11	(5)	1					1	4	1			25	
TF-23			2	5	12	(1)				1	1	1			1	24	
TF-34		1	7	(9)	2					1	3	1	1			25	
TF-46			5	12	1	◯				3	2	1	1			25	
TF-47		2	2	(6)	1	1				3	6	2				23	
TF-6	*ehd1ehd1*									2	9	(7)	2	2	1	23	46
TF-9											2	(14)	4	3		23	
TF-22										5	(4)	9	3		1	22	
TF-35									1	2	6	(10)		3	2	24	

[a] TF1 ~ TF152 = F_3 lines

[b] *Ehd1* and ehd1 are early and late heading alleles at *Ehd1* locus, respectively

[c] Circle indicates days to heading of each F_2 individual

[d] *Ehd1Ehd1* : *Ehd1ehd1* : *ehd1ehd1* = 35:71:46, $\chi^2 = 2.250$, $P = 0.324$)

Table 2 The basic vegetative growth (BVG) period and genotype of *Ef1* locus in three T65 isogenic lines and four Taiwanese rice varieties

Line	BVG (±SD)[a]	Genotype	Origin and cross combination
		Ef1 (=*Ehd1*)	
Taichung 65 (T65)	71.8 (±0.96) a	*ef1* (=*ehd1*)	Kameji/Shinriki
T65Ea	n.d.	*Ef1-a*	Taitung-tsailai
T65Eb	47.7 (±0.58) b	*Ef1-b*	Bozu 5
Tainung 70 (TU70)	76.5 (±2.08) c	*ef1* (=*ehd1*)	Tainung 67//Tainung 62/CNGY 243
Tainang 5 (TN5)	76.8 (±1.16) c	*ef1* (=*ehd1*)	Kaohsiung 18/Chinan 8
Tainang 7 (TN7)	70.3 (±0.96) a	*ef1* (=*ehd1*)	Nankaiyu 119/Nankaiyu 121
Tainang 9 (TN9)	83.9 (±4.96) d	*ef1* (=*ehd1*)	Hsinchugyu 467/Tainanggyu 162

n.d.: no data

[a] Means followed by the same letter are not significantly different according to Student's *t* test at $P = 0.05$

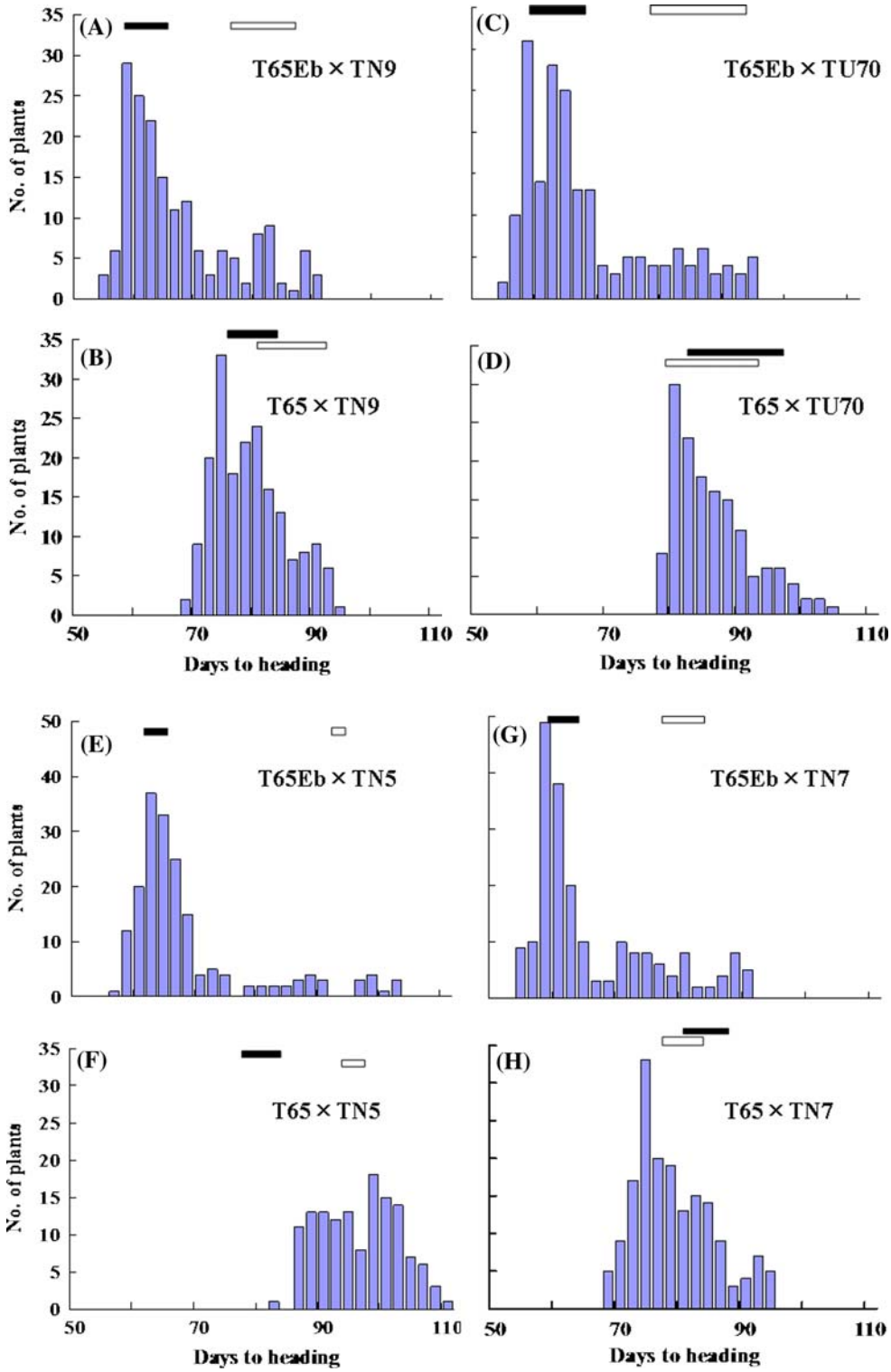

Fig. 2 Frequency distributions of days to heading in two F_2 populations, one from the cross of T65Eb × Taiwanese varieties **a**, **c**, **e**, and **g**, and one from the cross of T65 × Taiwanese varieties **b**, **d**, **f**, and **h**, under a 10 h photoperiod condition. A *white box* indicates the range of days to heading in T65Eb (**a**, **c**, **e**, and **g**) and T65 (**b**, **d**, **f**, and **h**). A *black box* indicates the range of days to heading in Taiwanese varieties

were significant but small or non-significant differences (0.3–13.6 days) among TN5, TN7, TN9, and TN70. We attempted to ascertain whether the long BVG periods of these four varieties are conferred by *ef1*. In all F_2 populations from the crosses between Taiwanese varieties and T65Eb (Fig. 2a, c, e, g), early (T65Eb type with a short BVG period) plants appeared at a high frequency under a 10 h photoperiod. In contrast, none of the F_2 populations of the crosses with T65 (Fig. 2b, d, f, h) produced any early (T65Eb-type) plants. These results

indicated that all four of the Taiwanese varieties carry *ef1* at the *Ef1* locus.

In the CAPS analysis, T65Eb (*Ef1-bEf1-b*) was observed to show the same banding pattern as Nipponbare, which indicates that *Ef1-b*, like *Ehd1-Nip*, harbors one *Dde*I restriction site in the GARP DNA-binding domain and functions to accelerate heading by reducing the BVG period (Fig. 3). In contrast, TN5, TN7, TN9, and TU70 showed the same banding pattern as T65. This indicates that these four varieties, like T65, carry *ef1* at the *Ef1* locus (Fig. 4).

Distribution of *ehd1* (=*ef1*) alleles among Asian rice varieties (Experiment 3)

In addition to the five varieties discussed above, 85 other varieties were also examined in order to determine each one's number of *Dde*I restriction sites and thus to understand the distribution of *ehd1* (=*ef1*) among rice varieties grown in south-eastern Asia. Out of 50 Taiwanese *japonica* varieties, 46 harbored two *Dde*I restriction sites, indicating that *ehd1* (=*ef1*) is widely distributed among Taiwanese *japonica* varieties (Table 3). In contrast, the remaining 40 south-eastern Asian varieties, including 7 Indonesian varieties, 10 Bangladeshi varieties, 21 Chinese varieties, and 2

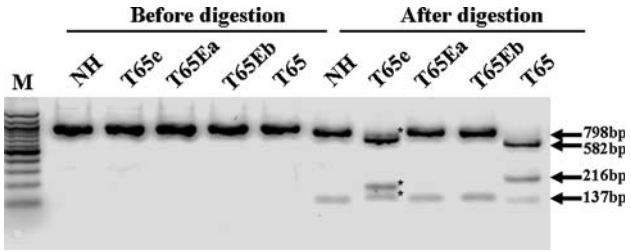

Fig. 3 CAPS analyses for the *Ehd1* alleles. *Arrows* indicate fragment sizes of digested amplicons estimated from the sequence data of *Ehd1-Nip* (AB092506) and *ehd1-T65* (AB092507). *Asterisk Ehd1-gla*, which has an additional restriction site not found in *Ehd1-Nip*, produced three fragments, containing 648, 150, and 137 bp, respectively. *M* 100 bp ladder, *NH* Nipponbare (*Ehd1-Nip*), *T65* Taichung 65 (*ehd1-T65*), *T65Ea and T65Eb* early heading isogenic lines of T65, *T65e* an early heading isogenic line of T65 (*Ehd1-gla*)

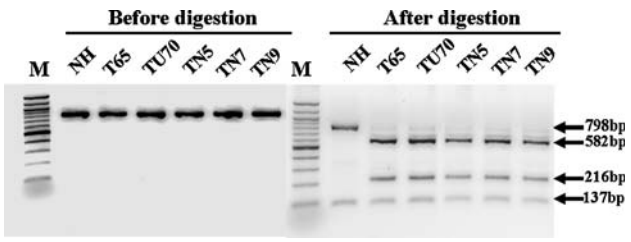

Fig. 4 CAPS analyses for the *Ehd1* alleles of four Taiwanese varieties. *M* 100 bp ladder, *NH* Nipponbare (*Ehd1-Nip*), *T65* Taichung 65 (*ehd1-T65*), *TU70* Tainung 70, *TN5* Tainang 5, *TN7* Tainang 7, and *TN9* Tainang 9

Table 3 Number of varieties and the genotype of CAPS analysis

Variety group	Number of varieties	The genotype according to CAPS analysis	
		Nipponbare-type	T65-type
Taiwanese varieties	50	4	46
Indonesian varieties	7	7	0
Bangladeshi varieties	10	10	0
Chinese varieties	21	21	0
IRRI varieties	2	2	0
Total	90	44	46

IRRI varieties, harbored only a single *Dde*I restriction site (Table 3). This may suggest that the *ehd1* (=*ef1*) allele is distributed only among the Taiwanese varieties.

Sequence analysis of *ef1-h* allele (Experiment 4)

Nishida et al. (2002) demonstrated that the late heading of the mutant line HS169 is governed by the mutant allele *ef1-h*, which prolongs the BVG period even more than *ef1* does. In contrast, HS169's original variety Gimbozu carries *Ef1*, as does Nipponbare. We compared the DNA sequence of *ef1-h* with that of *Ef1*. The results showed that the only difference between *ef1-h* in HS169 and *Ef1* was that the former harbors an insertion sequence of the transposon *mPing* in exon 2 (Fig. 5a GeneBank accession no. AK477988). The *mPing* sequence includes a stop codon at the 3′ terminal region (Nakazaki et al. 2003); hence the insertion of the *mPing* sequence does not provide the mature protein with the receiver domain and the GARP DNA-binding domain (Fig. 5b, c).

Discussion

Conventional genetic analysis demonstrated that *Ehd1* and *Ef1* are the same locus on chromosome 10; this conclusion was confirmed by CAPS analysis based on the number of *Dde*I restriction sites in the GARP DNA-binding domain in the middle part of *Ehd1* (Doi et al. 2004). It is therefore concluded that *Ef1-a*, *Ef1-b*, *Ehd1-Nip*, *Ehd1-gla*, *ef1*, and *ef1-h* are alleles at the *Ef1* (=*Ehd1*) locus: the first four are functional alleles, whereas the last two are non-functional. The CAPS analysis also showed that *Ef1-a, Ef-1b* and *Ehd1-Nip* have the same nucleotide sequence, implying that they are the same allele. In contrast, *Ehd1-Nip* and *Ehd1-gla* differ slightly in their nucleotide sequences (Doi et al. 2004); hence they might exhibit different accelerating effects on flowering. The difference accelerating effects of these two alleles should be further investigated.

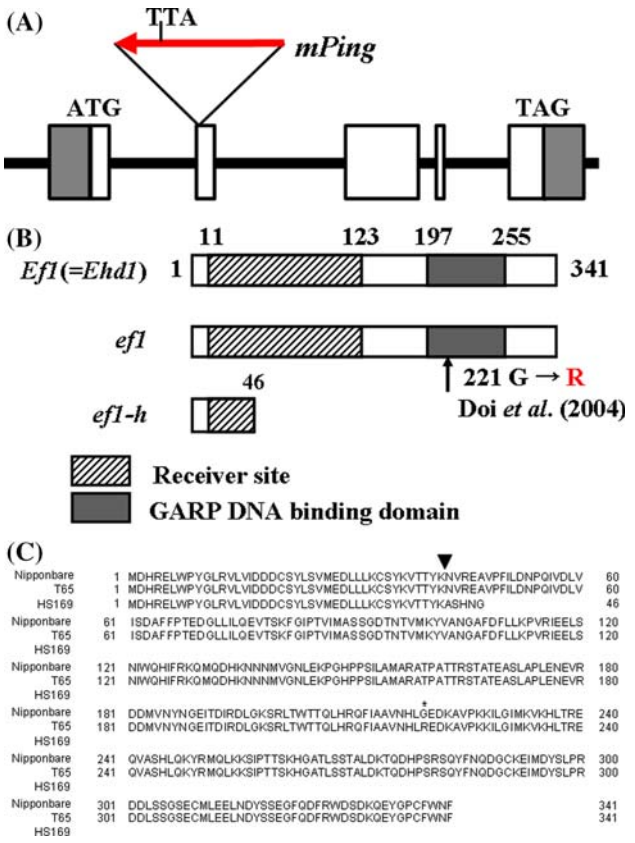

Fig. 5 Structure of *Ef1* (*Ehd1*). **a** Schematic representation of the genomic sequence of the *ef1-h* allele. *Open box* indicates the exon of the *Ef1* (*Ehd1*) gene. The position of an inserted *mPing* is shown with an *arrow*. **b** Protein structures of Nipponbare allele (*Ehd1-Nip*), T65 allele (*ef1*), and HS169 allele (*ef1-h*). The *stripe box* and *gray box* indicate a receiver site and a GARP DNA-binding domain, respectively. HS169 has a premature stop codon in the insertion of *mPing*. **c** Comparison of *Ehd1* amino acid sequences of Nipponbare (BAC77078), T65 (BAC77079) and HS169 alleles. *Arrowhead* indicates the *mPing* insertion site. *Asterisk* indicates the substitution site of T65 allele

The Taiwanese *japonica* varieties, TN5, TN7, TN9, and TU70, all exhibited extremely long BVG periods. Conventional genetic analysis showed that they all carry *ef1* at the *Ef1* locus; again, this conclusion was confirmed by the CAPS analysis. The rice varieties predominantly cultivated in Taiwan (21–26°N) were *indica* varieties until the first decade of the twentieth century when Japanese *japonica* varieties from the southern or middle part of Japan (25–39°N) were introduced. Because a large majority of the introduced Japanese varieties were strongly photoperiod-sensitive, their introduction to Taiwan did not improve Taiwanese rice production. Instead, the strong photoperiod sensitivity of the Japanese varieties inevitably resulted in early heading before the plants had achieved sufficient vegetative growth, resulting in low grain-yield performance (Iso 1963). This problem was overcome in 1936, however, with the development and release of the T65 variety, derived from the cross between two Japanese *japonica* varieties, Kameji and Shinriki, with an extremely long BVG period and weak photoperiod sensitivity. Since then, T65 has been used as a cross parent in the development of many elite *japonica* varieties that are well adapted to the climatic conditions and especially to the photoperiods found in Taiwan (11.0–13.0 h), and Taiwan now reliably produces *japonica* rice with a high grain yield. According to Lin (1991a, b), of the 99 *japonica* varieties developed between the 1940's and the 1980's, 80 were descendants of T65, and most of them exhibited an extremely long BVG period. This suggests that *ef1* is widely distributed among Taiwanese *japonica* varieties, and has made a significant contribution to rice production in Taiwan.

Our CAPS analysis showed that *ef1* is not present in any variety that is not Taiwanese origin. In addition, none of the Japanese varieties that have been investigated to date show an extremely long BVG period (Oka 1958; Sakamoto and Toriyama 1967; Sato and Hayashi 1985), suggesting that *ef1* is not distributed among Japanese *japonica* varieties. Thus, we conclude that the *ef1* allele is peculiar to Taiwanese rice varieties. As described above, T65 was developed with the cross between two Japanese *japonica* varieties, Kameji and Shinriki. As we have reported in previous studies, however, Shinriki harbors *Ef1* at the *Ef1* locus (Okumoto et al. 1992a). Furthermore, it is assumed that Kameji also harbors *Ef1* at the *Ef1* locus because it exhibits a short BVG period, equivalent to that of Shinriki, although detailed investigations into the Kameji genotype have not been conducted. Therefore, there is a high possibility that *ef1* was a spontaneous mutant gene, which was probably induced in the process of developing T65.

Although *ef1* increases the BVG period on its own, its effect can be enhanced by the complementary effect of the nonfunctional allele *Se1-e* at the *Se1* (*Photoperiod sensitivity-1*) locus (Nishida et al. 2001; Ichitani et al. 2002). The mutant allele *ef1-h*, on the other hand, confers an extremely long BVG period without the aid of any other non-allelic genes, including *Se1-e*. In addition, the effect of *ef1-h* is much greater than the complementary effect of *ef1* and *Se1-e*. Based on these results, Nishida et al. (2002) proposed that *ef1* permits some residual function to accelerate heading, while *ef1-h* causes the complete loss of that function. The sequence analysis of *ef1-h* revealed that this allele did not harbor a Gly to Arg substitution in the GARP DNA-binding domain, but instead had an insertion sequence of *mPing* in exon 2. Except for this *mPing* insertion, *ef1-h* has the same sequence as *Ef1*. It is therefore considered that the complete loss of function caused by *ef1-h* relies on the insertion of *mPing*.

As described above, *ef1* does not exist in Asian *indica* and tropical *japonica* varieties. Numerous elite rice varieties with the extremely long BVG period and weak

photoperiod sensitivity have been developed for low latitude regions, which may suggest different BVG genes were utilized. To date, three BVG genes, *ef3*, *ef4*, and *ef5,* have been identified in *indica* varieties, and one BVG gene was identified in a tropical *japonica* variety; some of these four genes might have contributed to the development of the new elite varieties for the low latitudes. Both *ef1* and *ef1-h* are non-allelic to the four genes mentioned above; therefore, they represent novel gene sources that can aid in the development of elite *indica* and *japonica* varieties for the low latitudes.

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