Control of Awn Length in Rice Breeding Programs in Hokkaido

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Abstract

Understanding genetic diversity is a primary goal of the molecular evolution of the genes. Awn length is a well-documented phenotype among domestication traits in rice, from long to short awn. In addition, awnlessness is favor for current rice farmers. Here, we identified the genetic basis of awn length during rice breeding programs in Hokkaido. We found variation of awn length ranging from 0.0 to 37.6 mm. Varieties with a short-awn or awnlessness have been selected under rice breeding programs. Genetic analysis on awn length identified that *RAE1* and *RAE2* on chromosomes 4 and 8, respectively, accounted for awning. These genes were well known to be significant during Asian rice domestication. Sequence variations of the genes would clarify the molecular evolution of the genes on awn length. Firstly, the loss-of-function allele in *RAE1*, *rae1*, was selected for short awn length. Then, alleles on *RAE2*, *RAE2-H01* to *RAE2-H04*, were targeted for the selection of short-awn or awnlessness. The selections on awnlessness phenotype could diversify these alleles on the genes, *RAE1* and *RAE2*, exhibiting the variation of awn length.

Introduction

Understanding of the genetic diversity is a primary goal of the molecular evolution of the gene for sustainable plant breeding programs. Awn is a needle-like organ extending from the lemma tip of spikelet. Awns are characteristic of seeds of wild plants as they aid in seed dispersal (Elbaum et al. 2007; Guo and Schnurbusch 2016). They also contribute to the photosynthetic activity of the inflorescence in wheat and barley, though not in rice (Rebetzke et al. 2016). Awn length of the short awn is a domestication trait in rice. Many genes for awn length have been characterized, some at the molecular level (Bessho-Uehara et al. 2016; Furuta et al. 2015; Hua et al. 2015; Luo et al. 2013; Zhang et al. 2019); those related to short awn length include *Awn-1* (*An-1*), *Awn-2* (*An-2*), *LONG AND BARBED AWN 1* (*LABA1*), *Regulator of Awn Elongation 1, 2, and 3* (*RAE1*, *RAE2*, *RAE3*), and *Grain Length and Awn Development* (*GLA*). Whereas awnlessness is one of the major targets for rice breeding programs. Awn could inhibit the handling of rice seed in rice cultivation. Awnlessness is favor for current rice farmers.

Asian cultivated rice, *Oryza sativa* L., is a major staple food that provides the caloric requirements for the world's population and originated in the tropics. Rice cultivation have increased rice production under various climatic conditions at latitudes between 53°N and 40°S (Lu and Chang 1980). In local rice areas, various kinds of traits might be successful under artificial selection to establish rice cultivation. For example, those in Japan focus on eating quality and the adaptability to local environmental conditions (Fujino et al. 2019a; Kobayashi et al. 2018). Recent molecular genomics seek to understand the adaptability of historical processes in modern crop breeding programs. The genetic and phenotypic diversity on the traits has been characterized among varieties during rice breeding programs (Fujino et al. 2015, 2017, 2019a; Shinada et al. 2014). However, the genetic basis of the selections has not been clarified.

Dysfunctional alleles of *RAE1* (on chromosome 4) and *RAE2* (on chromosome 8), which are involved in the genes for the control of awn length have been selected during Asian rice domestication (Bessho-Uehara et al. 2016). Comparative analysis of *RAE1* and *RAE2* revealed that a two-step loss of function contributed to awn length (Bessho-Uehara et al. 2021). Loss-of-function alleles of both genes, *rae1* and *rae2*, are typical in *japonica* rice (Bessho-Uehara et al. 2021). *RAE1* encodes a bHLH transcription factor (Luo et al. 2013). *RAE2* encodes *Epidermal Patterning Factor-Like protein 1* (Bessho-Uehara et al. 2016). Four haplotype combinations between *RAE1* and *RAE2*, groups I-IV, have been characterized for the genetic diversity of the sequence among Asian cultivated rice (Bessho-Uehara et al. 2021).

This study focused on the variation of awn length on the transition from long awns to short awns or awnlessness during rice breeding programs in Hokkaido. Genetic analysis identified that *RAE1* and *RAE2* contribute to awn length including awnlessness. To clarify the selection history of awn length, we searched for the sequence variations of *RAE1* and *RAE2* and phenotype of awn length. We could elucidate the genetic base for the control of awn length in rice breeding programs in Hokkaido.

Materials And Methods

Plant materials

‘Akage’ and ‘Kitaibuki’ were used as parents for linkage analysis for awn length. ‘Akage’ is a long-awned landrace from Hokkaido, Japan, at one of the northern-limits of rice cultivation in the world (Fujino et al. 2017, 2019a). A rice variety ‘Kitaibuki’ is awnless and was registered in 1990. To identify QTLs controlling awn length, we developed an F₂ population ($n = 181$) derived from a cross between them. In brief, F₁ plants were self-pollinated to produce F₂ seeds.

We compared sequences of genes for awn length among varieties from three populations. One was the Hokkaido Rice Core Panel (HRCP) to identify historical changes in awn length during rice breeding programs in Hokkaido (Shinada et al. 2014). Second was 44 Hokkaido Landraces (HL), which was ancestral varieties for the HRCP (Fujino et al. 2019). Third was 45 varieties from the Japanese Rice Core Collection (JRC), which represents genetic diversity among the ancestral gene pool of varieties in Japan (Ebana et al. 2008; Tanaka et al. 2021).

Seeds were provided by the Genebank of NARO (Tsukuba, Japan) and the Local Independent Administrative Agency, Hokkaido Research Organization, Hokkaido Central Agricultural Experiment Station (Takikawa, Japan).

Measurement of awn length

All plant materials were grown in an experimental paddy field at Hokkaido Agricultural Research Center (Sapporo, Hokkaido, Japan, 43°00'N) in 2015 (Fujino et al. 2018). Awn length was measured as the average awn length of the apical spikelet of each primary branch on a panicle of the longest culm on each of three plants (Hua et al. 2015; Luo et al. 2013). Means and SDs of triplicates are shown.

Differences between means were tested by two-way analysis of variance (ANOVA) and by Tukey–Kramer HSD test to show epistatic interactions between genes.

DNA analysis

Seeds from Genebank were sown for DNA isolation without propagation. Total DNA was isolated from young leaves by the CTAB method (Murray and Thompson 1980). PCR and sequencing were performed as described by Fujino et al. (2004, 2005). Primers for genotyping of chromosomal regions for *RAE1* and *RAE2* in the F_2 population are listed in Supplementary Table 1. These indel markers were developed by the myINDEL procedure (Fujino et al. 2018).

We compared the *RAE1* and *RAE2* sequences. PCR experiments to test for the presence or absence of a transposon insertion in *RAE1* were carried out using three primer pairs that target the transposon and the genomic regions flanking the insertion of the transposon (Supplementary Fig. 1, Supplementary Table 1). The transposon insertion causes loss-of-function of the gene (Luo et al. 2013).

The genomic region of *RAE2*, including the upstream region, coding region, and downstream region, was amplified and sequenced.

DNA sequences were initially aligned in BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and then adjusted by eye. All polymorphisms were re-checked from chromatograms with special attention to low-frequency polymorphisms. Heterozygosity was not observed.

Sequence data for haplotyping around *RAE1* and *RAE2* are drawn from the SRA/ENA/DRA databases under accession numbers DRA006061 and DRA008447.

Results

Variation in awn length among HRCP cultivars

Awn length varied among varieties in HRCP, from 0.0 mm in 26 varieties, which were breeding lines, to 37.9 mm in ‘Akage’ (Fig. 2, Supplementary Table 2). Among seven landraces, it ranged from 0.0 mm in ‘Bouzu’ and ‘Wasebouzu’ to 37.9 mm in ‘Akage’ (Supplementary Table 2). Awns longer than 3.5 mm were not observed in any of the 23 varieties bred since 1975 (Supplementary Table 2). This phenotypic change on awn length might be derived from the genes for the trait during rice breeding programs.

Two chromosomal regions for awn length

To identify chromosomal regions controlling awn length, we carried out genetic analysis using an F_2 population derived from ‘Akage’ (39.9 ± 8.9 mm) $\times$ ‘Kitaibuki’ (0.0 ± 0.0 mm). Awn length in the F_2 population varied widely with a continuous distribution, from 0.0 to 54.9 mm (Fig. 3A). Next, to identify the genetic bases of this awn length regulation, association of the awn length was examined. It is known

that *RAE1* and *RAE2* contribute for the control of awn length in Asian cultivated rice (Bessho-Uehara et al. 2016). A clear association between awn length and the genotypes of the marker AwnAKKT102 on chromosome 4 (QTL1) near to *RAE1* and the marker AwnAKKT204 on chromosome 8 (QTL2) near to *RAE2* (Furuta et al. 2015) was determined among the F2 population (Fig. 3, Supplementary Table 3). We concluded that *RAE1* and *RAE2* accounted for the awn length.

According to the genotype of two QTLs, QTL1 and QTL2 for *RAE1* and *RAE2*, respectively, plants in this population were classified into nine genotype classes (Fig. 3B, Supplementary Table 3). The awn lengths of each genotype class were significantly different. Plants homozygous for the Kitaibuki alleles at both genes, were 0.8 ± 2.3 mm (range 0.0–7.3 mm). Whereas plants with the Akage alleles at both genes had long awns of 44.0 ± 8.4 mm (range 35.7–54.9 mm). The Akage alleles at either QTL1 nor QTL2 showed awn lengths of 23.4 and 19.1 mm, respectively. The Akage alleles at each locus were dominant with an effect of enhancing awn length. These two awn genes had additive effects on awn length (Fig. 3B, Supplementary Table 3). The genetic base identified in this study was consisting with the results in cultivated-wild rice study as rice domestication (Bessho-Uehara et al. 2016; Furuta et al. 2015).

Sequence variations in *RAE1*

Next, we identified sequence diversity in *RAE1* and *RAE2* for awn length. The transposon insertion in *RAE1* caused loss-of-function allele, *rae1* (Luo et al. 2013). We used the PCR procedure to survey the presence/absence of the transposon (Supplementary Fig. 1) in the three populations, HRCP, HL, and JRC (Table 1). Among HRCP, all breeding lines, except for a single variety Norin No. 34, carried the transposon insertion allele, *rae1* (Supplementary Table 2). Whereas 30 of 44 HL varieties and 33 of 45 JRC varieties carried the transposon insertion allele (Supplementary Tables 4, 5). These results suggest that the functional allele conferring the awned phenotype is maintained among the populations, HL and JRC, which might provide a morphological marker for distinguishing varieties before rice breeding programs on scientific theory (Table 1).

Sequence variations in *RAE2*

Next, sequences of *RAE2* in HRCP were compared with the *RAE2* reported in Bessho-Uehara et al. (2021). In addition to the 6-bp insertion in all HRCP varieties, we identified four polymorphisms (Supplementary Fig. 2; Supplementary Table 2). We could identify four alleles, *RAE2-H01*~*RAE2-H04*. *RAE2-H01* had no polymorphisms. *RAE2-H02* had a 6-bp deletion generating a 2-amino-acid deletion and a 1-bp polymorphism generating a 1-amino-acid substitution. *RAE2-H03* had a 2-bp deletion generating a frame-shift in translation. *RAE2-H04* had a 4-bp deletion generating a frame-shift in translation. By the polymorphisms in the GC-rich region in *RAE2*, *RAE2-H01*, *RAE2-H03*, and *RAE2-H04* identified in this study were identical to *RAE2*-hap 1, 5, and 3, respectively, of Bessho-Uehara et al. (2021). *RAE2-H02* was novel allele to Bessho-Uehara et al. (2021).

The selections of the *RAE2* allele seemed to be along with the process of rice breeding programs (Table 2). In the initial phase, there was *RAE2-H01*, which is functional allele. Then, *RAE2-H02* and *RAE2-H03*

were selected and appeared. Finally, *RAE2-H04*, was detected at two varieties, ‘Shimahikari’ and ‘Nanatsuboshi’, which were bred in 1981 and 2001, respectively (Supplementary Table 2).

Haplotypes around RAE1 and RAE2

To clarify the selection signature on these alleles for awn length, we compared the genotype surrounding chromosomal regions (± 100 kb) of these alleles in *RAE1* and *RAE2* in 26 varieties (Fig. 4, Table 3). In the *RAE1* region, we identified three major haplotypes, Hap=RAE1=H01 to H03. Hap=RAE1=H02 was divided into three sub-haplotypes, H02a to c. Hap=RAE1=H03 was divided into two sub-haplotypes, H03a and b. Both *RAE1* (identified by “ntp” in Fig. 4A) and *rae1*, which was the transposon insertion allele (“T”), were present in Hap=RAE1=H01. Whereas Hap=RAE1=H02 and Hap=RAE1=H03 included only *rae1*. These clearly indicated that the transposon insertion causing loss-of-function in *RAE1* occurred in Hap=RAE1=H01. Then, the loss-of-function allele, *rae1*, might be recruited into the haplotypes, Hap=RAE1=H02 and Hap=RAE1=H03.

Different combinations of *RAE1* alleles and haplotypes suggested that there were introgressions with crossing-over of micro-chromosomal segments (Fig. 4A). The loss-of-function allele, *rae1*, was found in five haplotypes, Hap=RAE1=H01, H02a–c, and H03b (Fig. 4A). A chromosomal segment around *rae1* was introgressed from Hap=RAE1=H01 into Hap=RAE1=H02 and H03, which were up to 7806 bp between flanking SNPs at the positions of 16 740 388 and 16 732 582 bp (Supplementary Table 6).

In the *RAE2* region, we identified three major haplotypes, Hap=RAE2=H01 to Hap=RAE2=H03 (Fig. 4B, Supplementary Table 7). Hap=RAE2=H01 was divided into five sub-haplotypes, Hap=RAE2=H01a to Hap=RAE2=H01e. Four alleles in *RAE2* correlated well with haplogroups (Fig. 4B). Allele *RAE2-H01* was present in Hap=RAE2=H01a, c, and e. *RAE2-H02* was in Hap=RAE2=H01d. *RAE2-H03* was in Hap=RAE2=H02. *RAE2-H04* was in Hap=RAE2=H03.

Discussion

Recent molecular evidence was targeted on the domestication traits including awn phenotype, long awn in wild and awnless in cultivated in rice (Bessho-Uehara et al. 2016; Furuta et al. 2015; Hua et al. 2015; Luo et al. 2013; Zhang et al. 2019). The molecular mechanisms involved in variation of awn length among varieties in rice breeding programs have been unclear. Here, we focused on the controlling of awn length among varieties in rice breeding programs in Hokkaido. Genetic analysis in this study revealed that two genes, *RAE1* and *RAE2*, contribute to the controlling of awn length. Sequence variations in *RAE1* and *RAE2* revealed the diversification of alleles in the genes under the selection history of awn length during rice breeding programs. At first, *RAE1* was under the selection. The loss-of-function allele of *RAE1* as the transposon insertion allele, *rae1*, was selected. This allele deduced awn length. Then, *RAE2* was under the selection (Table 2), resulting in shorter awn length and awnlessness, *rae1 rae2* (Table S2).

Diversity in *RAE1* and *RAE2*, which were already characterized as domestication genes (Bessho-Uehara et al. 2016), found in this study revealed the dynamics of molecular evolution of the gene. *RAE1/An-1* was

divided into two major haplotypes between cultivated rice and wild rice, and the haplotype in cultivated rice classified into two sub-haplotypes (Luo et al. 2013). Our results show that awned varieties carry *RAE1* (without transposon insertion), whereas short-awned or awnless varieties carry *rae1* (with transposon insertion). We found both two alleles, *RAE1* and *rae1*, in a single local population. While we found four alleles at *RAE2*, *RAE2-H01* to *H04*. Genes for awn length have pleiotropic effects on yield traits (Bessho-Uehara et al. 2016; Gu et al. 2015; Hua et al. 2015; Jin et al. 2016; Luo et al. 2013). The four *RAE2* alleles might exhibit pleiotropic effects on yield traits during rice breeding programs, which showed the genetic shifts (Shinada et al. 2014).

We here propose a model of molecular evolution in *RAE1* (Fig. 5). Two haplotypes with a functional *RAE1*, Hap=*RAE1*=H01 and Hap=*RAE1*=H03 were ancestral. Transposon insertion into *RAE1* in Hap=*RAE1*=H01 generated *rae1*. Then, *rae1* might recruited into two haplotypes, Hap=*RAE1*=H02 and Hap=*RAE1*=H03. The introgressions of *rae1* appear to have been generated from crossing-over of micro-chromosomal segments within a region of up to 7806 bp between haplotypes. Due to the crossing-over, we found five combinations of *RAE1* alleles with haplotypes around *RAE1*.

The *RAE1/An-1* locus might have undergone multiple recombination events or genetic drift during domestication (Luo et al. 2013). The gene flows for short-awn or awnlessness between *japonica* and *indica* cultivars has been shown (Zhang et al. 2019). Within 60 years in rice breeding programs in Hokkaido, from the start of the programs in 1915, awn was completely lost on both phenotype and genotype on *RAE1* and *RAE2*. During rice breeding programs in Hokkaido, genomic changes have been characterized (Fujino et al. 2015, 2021; Shinada et al. 2014). Our findings of *rae1* gene flows and multiple *rae2* alleles clearly demonstrated that rice breeding programs could generate genetic diversity among self-pollinating plant. Deep understanding of genetic base of the controlling awn length on *RAE1* and *RAE2* under rice breeding programs could open the door to molecular evolution in the desirable genes.

Declarations

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Author contribution statement

KF conceived and designed the experiments and wrote the manuscript. All authors performed the experiments, analyzed the data, and approved the final manuscript.

Data availability Materials reported in this study, phenotype and genotype data of mapping populations, are available upon request.

Ethics approval Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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Tables

Table 1 to 3 are only available as a download in the Supplemental Files section.

Figures

Figure 1

Awns on (A) panicles and (B) spikelets. Top (in A) and left (in B): HRCP 26 (Norin No. 34); middle: HRCP 34 (Kitakoganne); bottom and right: HRCP 10 (Norin No. 9). Scale bars 20 mm in A and B.

Figure 2

Variation in awn length among the HRCP population. The dashed vertical line separates landraces (LR) before the breeding programs began and breeding lines after they began. Year indicates the year of registration of the varieties.

Figure 3

Variation in awn length by genotype at two chromosomal regions among the F_2 population ($n = 181$) derived from 'Kitaibuki' (KT) $\times$ 'Akage' (AG). A: Frequency distributions of awn length; vertical and horizontal bars show the mean and range, respectively, of awn length in the parents. B: Awn length in nine genotypic classes; A, 'Kitaibuki'; B, 'Akage'; H, heterozygous; Kitaibuki is AA and Akage is BB. Bars with the same letters are not significantly different at $p < 0.05$.

Figure 4

Definition of haplotypes around (A) *RAE1* and (B) *RAE2* within ± 100 kb. Direction of arrow above the gene (●) indicates the strand coding the gene. Horizontal line indicates chromosome. Vertical bars show positions of SNPs. Haplotypes are indicated at the left. "Genotype" (at right in A): T, transposon-inserted; ntp, not inserted. Color is specific to haplotype. "Allele" (at right in B): allele in *RAE2*.

Figure 5

Model of molecular evolution in *RAE1* on chromosome 4. In the ancestral gene pool, the functional allele, *RAE1*, is present in Hap=*RAE1*=H01 (black line) and Hap=*RAE1*=H03 (light gray line). Insertion of the transposon (triangle) into *RAE1* generated the loss-of-function allele *rae1*. *rae1* on Hap=*RAE1*=H01 was introgressed into Hap=*RAE1*=H02 (dark gray line) and Hap=*RAE1*=H03. Gray box indicates the genotype at the site of 16,732,582 in the *RAE1* region. Hap=*RAE1*=H02 carries *rae1* with a single nucleotide substitution, T→A.

Supplementary Files

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