Control of awn length in rice breeding programs in Hokkaido

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Abstract Understanding genetic diversity among local populations may facilitate the development of new crop varieties and is a primary goal of the molecular evolution of genes. Awn length is a well-documented phenotype among domestication traits in rice (*Oryza sativa* L.), from long to short awns. Short awned or awnless varieties have been selected in rice breeding programs. Awnlessness is favored by rice farmers in the current agriculture system. Here, we identified the genetic basis of awn length during rice breeding programs in Hokkaido, Japan. We found variation in awn length ranging from 0.0 to 37.6 mm among a local population consisted of breeding lines. Genetic analysis of awn length identified that RAE1 and RAE2 on chromosomes 4 and 8, respectively, accounted for awn presence. These genes are well known to be significant during Asian rice domestication. Sequence variations in these genes may clarify the molecular evolution of the genes for awn length in rice breeding programs. Firstly, a loss-of-function allele in RAE1, rae1, was selected for short awn length. Then, alleles of RAE2, RAE2-H01 to RAE2-H04, were targeted for the selection of short awns or awnlessness. The selections of an awnlessness phenotype can diversify these alleles in the genes RAE1 and RAE2 exhibiting variation in awn length.

Keywords Awn · Rice · Rice breeding programs · Selection

Introduction

Understanding of genetic diversity is a primary goal in the study of molecular evolution of genes for sustainable plant breeding programs. Awns are a needle-like organ extending from the lemma tip of a spikelet, and which aid in seed dispersal in wild plants (Elbaum et al. 2007; Guo and Schnurbusch 2016). They also contribute to the photosynthetic activity of inflorescences in wheat and barley, though not in rice.
Short awn length is a domestication trait in rice. Many genes for awn length have been characterized including 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) (Bessho-Uehara et al. 2016; Furuta et al. 2015; Hua et al. 2015; Luo et al. 2013; Zhang et al. 2019). Awnlessness is one of the major targets for rice breeding programs. Awns can inhibit the handling of rice seeds in rice cultivation; therefore, awnlessness is favored by rice farmers in the current agricultural system.

Asian cultivated rice, *Oryza sativa* L., is a major staple food that provides the caloric requirements for a large proportion of the world’s population. Although rice originated in the tropics, its cultivation has increased in 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 stable rice cultivation. For example, those in Japan focus on eating quality and the adaptability to local environmental conditions (Fujino et al. 2019; Kobayashi et al. 2018). Recent molecular genomics analyses have sought to understand the adaptability of historical processes in modern crop breeding programs (Fujino et al. 2019; Shinada et al. 2014). However, the genetic basis of selection has not been clarified.

Dysfunctional alleles of RAE1 (chromosome 4) and RAE2 (chromosome 8), which are genes involved in 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 basic helix-loop-helix 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 sequences among Asian cultivated rice (Bessho-Uehara et al. 2021).

This study focused on the variation in 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 sequence variations in RAE1 and RAE2 and the phenotype of awn length. We were able to elucidate the genetic basis for the control of awn length in rice breeding programs in Hokkaido.

**Materials and methods**

**Plant materials**

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

Sequences of genes for awn length were compared among varieties from three populations. First, the Hokkaido Rice Core Panel (HRCP) was used to identify historical changes in awn length during rice breeding programs in Hokkaido (Shinada et al. 2014). Second was 45 Hokkaido Landraces (HLs), which were ancestral varieties for the HRCP. Third was 50 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. 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 standard deviations (SDs) of measurements in triplicate are shown for awn length of the varieties. Differences between means of awn length were tested using 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 using the CTAB method (Murray and Thompson 1980). PCR and sequencing were performed as described by Fujino et al. (2004). Primers for genotyping of the chromosomal regions for RAE1 and RAE2 in the F2 population are listed in Supplementary Table 1. These indel markers were developed using the myINDEL procedure (Fujino et al. 2018).

The RAE1 and RAE2 sequences were compared among HRCP varieties. PCR experiments to test for the presence or absence of a transposon insertion in RAE1 were carried out using three primer pairs that target in the transposon and the genomic regions flanking the insertion site of the transposon (Supplementary Fig. 1, Supplementary Table 1). The transposon insertion in RAE1 causes loss-of-function of the gene, rae1 (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 using BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and then manually adjusted. All polymorphisms were rechecked from chromatograms with special attention to low-frequency polymorphisms. Heterozygosity was not observed in the experiment (Fig. 1).

**Results**

Variation in awn length among HRCP cultivars

Awn length varied among varieties in the HRCP, from 0.0 mm in 26 varieties as breeding lines, to 37.9 mm in ‘Akage’ (Figs. 1, 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

![Fig. 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](image_url)
were not observed in any of the 23 varieties bred since 1975 (Supplementary Table 2). Awn length has been selected for short awns and awnlessness.

Two chromosomal regions for awn length

To identify chromosomal regions controlling awn length, we carried out genetic analysis using an F2 population derived from ‘Akage’ (39.9 ± 8.9 mm) × ‘Kitaibuki’ (0.0 ± 0.0 mm). Awn length in the F2 population varied widely with a continuous distribution, from 0.0 to 54.9 mm (Fig. 3A). Next, to identify the genetic basis of awn length regulation, association of phenotype with awn length and genotype of genes for 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 detected among the F2 population (Fig. 3, Supplementary Table 3).

According to the genotype of two QTLs, QTL1 and QTL2, 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, had short awns of 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 long 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 basis identified in this study consisted of the results in cultivated-wild rice as part of rice domestication (Bessho-Uehara et al. 2016; Furuta et al. 2015).

Sequence variations in RAE1

Next, sequence diversity in RAE1 and RAE2 for awn length was identified. The transposon insertion in RAE1 caused loss-of-function of the gene, rae1 (Luo et al. 2013). A PCR procedure to survey the presence/absence of the transposon was used (Supplementary Fig. 1) in the three populations, HRCP, HL, and JRC (Table 1). Among the HRCP, all breeding lines except for a single variety, Norin No. 34, carried the transposon insertion allele of 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 populations, such as the HL and JRC, which might provide a morphological marker 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.
for distinguishing varieties before rice breeding programs with scientific theory (Table 1).

Sequence variations in RAE2

Next, sequences of RAE2 in the HRCP were compared with RAE2 reported by Bessho-Uehara et al. (2021). In addition to the 6-bp insertion, four polymorphisms were identified, RAE2-H01~RAE2-H04 (Supplementary Fig. 2; Supplementary Table 2). RAE2-H01 had a 6-bp insertion only. 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 frameshift in translation of RAE2. RAE2-H04 had a 4-bp deletion generating a frameshift in the translation of RAE2. RAE2-H01, RAE2-H03, and RAE2-H04 identified in this study were identical to RAE2-hap 1, RAE2-hap 5, and RAE2-hap 3, respectively, of Bessho-Uehara et al. (2021). RAE2-H02 was a novel allele compared with Bessho-Uehara et al. (2021).

The selection of the RAE2 allele seemed to occur along with the process of rice breeding programs (Table 2). In the initial phase of rice breeding programs in Hokkaido, there was RAE2-H01. Then, RAE2-H02 and RAE2-H03 were selected. Finally, RAE2-H04, was detected in two varieties, ‘Shimahikari’ and ‘Nanatsuboshi’, which were bred in 1981 and 2001, respectively (Supplementary Table 2).

Table 1  Distributions of the transposon-like elements inserted into RAE1 allele

| Population | N* | Number | Frequency (%) |
|------------|----|--------|---------------|
| HRCP       | 62 | 58     | 93.5          |
| HL         | 44 | 30     | 68.1          |
| JRC        | 45 | 33     | 73.3          |

*Total number of varieties in each population

Table 2  Distribution of RAE2 among HRCP populations

| Allele | n  | <1910 | <1950 | <1975 | <2010 |
|--------|----|-------|-------|-------|-------|
| H01    | 17 | 5     | 8     | 3     | 1     |
| H02    | 12 | 2     | 5     | 5     |       |
| H03    | 15 | 3     | 12    |       |       |
| H04    | 2  | 3     |       |       |       |

Discussion

Recent molecular evidence has targeted domestication traits including awn phenotype, with long awns in wild varieties and awnlessness 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). However, the molecular mechanisms involved in variation of awn length among varieties in rice breeding programs have been unclear. Here, we focused on the control of awn length among varieties in rice breeding programs in Hokkaido. Genetic analysis demonstrated that two genes, RAE1 and RAE2, contributed to the control of awn length (Fig. 3). Sequence variations in RAE1 and RAE2 revealed the diversification of alleles in the genes under the selection history.

Diversity in RAE1 and RAE2 revealed the dynamics of molecular evolution of the gene (Fig. S2). 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). Awned varieties carry RAE1 (without transposon insertion), whereas short-awned or awnless varieties carry rae1 (with transposon insertion). Four alleles at RAE2 were found, 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).

The combination of RAE1 and RAE2 in rice varieties in Hokkaido was loss-of-function, which corresponded to group IV defined in cultivated rice (Bessho-Uehara et al. 2021). Therefore, different alleles were in rae2. Within 60 years in rice breeding programs in Hokkaido, from the start of programs in 1915, awns were completely lost in the evaluation of both phenotype of awn length and genotype at RAE1 and RAE2. Awnlessness was under selection during rice breeding programs in Hokkaido (Fig. 2). The selection was carried out based on the phenotype of awn length. The selection also completed the genetic shifts in RAE1 and RAE2 (Fig. 4). First, RAE1 was under the selection from the long to short awns. Loss-of-function rae1 was selected. rae1 was distributed over the ancestral population of the HRCP, HL, and JRC. Intensive
Selection for short awns may have focused on \textit{rae1}. Then, \textit{RAE2} was under selection from short awns to awnlessness, as determined by \textit{RAE2-H01} to \textit{RAE2-H04}.

During rice breeding programs in Hokkaido, genomic changes have been characterized (Fujino et al. 2021; Shinada et al. 2014). The selection may drive genetic diversity among rice breeding programs in Hokkaido, involving a single \textit{rae1} allele and multiple \textit{RAE2} alleles. Deeper understanding of the genetic basis controlling awn length at \textit{RAE1} and \textit{RAE2} may open the door to controlling genetic diversity in rice breeding programs in future stages and in the molecular evolution of desirable genes.

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

Declarations

Competing interests The authors declare no competing interests.

Ethics approval Not applicable.

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