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Both *Hd1* and *Ehd1* are important for artificial selection of flowering time in cultivated rice

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ABSTRACT

Rice is a facultative short-day plant, and it requires a photoperiod shorter than the critical day length to get flowering. Sensitivity to photoperiod has been suggested as a major selection target in cultivated or weedy rice. The modern rice varieties in Taiwan may be cultivated twice a year. These varieties contain loss-of-function of two important flowering-time related genes, *Heading date 1* (*Hd1*) and *Early heading date 1* (*Ehd1*), and are mainly from a mega variety, Taichung 65. However, the parental lines of this variety were sensitive to photoperiod, thus, how Taichung 65 loss its sensitivity is a mystery. In this study, we used accession-specific single nucleotide polymorphism analysis to reveal the gene flow that occurred between different rice accessions decades ago and demonstrate that two landraces introgressed during the breeding process, which led to the loss of photoperiod sensitivity. Both *Hd1* and *Ehd1* may be important during artificial selection for flowering time, especially in a subtropical region such as Taiwan. This is a good example of introgression playing important roles during rice domestication.

1. Introduction

Introgression or hybridization may play an important role in crop domestication. For rice, gene flow and genetic isolation with several domestication-related genes has been used to reveal the dynamics of rice domestication [1,2]. One of the early domestication traits found was shattering (SH), a quantitative trait loci (QTL) that controls cell-wall degradation and therefore, established the abscission layer between the grain and panicle. *SH4* is a putative transcription factor [3] and *SH1* encodes a homeobox transcription factor [4]. Because *SH4* is present in many *indica* and *japonica* rice accessions, the *SH4* allele was considered to have appeared and been selected before the differentiation of *indica* and *japonica* from a common wild rice ancestor or selected very early during the domestication of one rice subpopulation and then introgressed to others. Heerwaarden and colleagues used single nucleotide polymorphism (SNP) datasets for a large number of accessions of both teosinte and maize and demonstrated gene flow between maize and its wild relatives [5]. A recent spontaneous DNA introgression was found from domesticated polyploid wheat into its distantly related, wild tetraploid *Aegilops peregrine,* with the stabilization of this sequence in wild populations despite their not having homologous chromosomes. Thus, DNA may spontaneously introgress between homoeologous genomes of species of the tribe Triticeae [6].

Flowering time, also known as heading date for rice, is an important trait during rice domestication [7]. Flowering time is affected by photoperiod (day length) and also temperature [8]. As a facultative short-day plant, rice flowering is promoted under short-day conditions and delayed under long-day conditions, with the critical day length around 13 h [9,10]. Many recent reviews provided detailed information on the regulation of rice flowering [11–13]. A few key genes have been suggested to affect flowering time,

Abbreviations: BVG, basic vegetative growth; FNP, functional nucleotide polymorphism; IGV, integrative genomics viewer; LTR, long terminal repeat; QTL, quantitative trait loci; SNP, single nucleotide polymorphism; TC65, Taichung 65; TNG67, Tainung 67; VCF, variant call format.

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including *Heading date 1* (*Hd1*), a B-box zinc finger protein and the ortholog of Arabidopsis CONSTAINS [14]; *Early heading date* (*Ehd1*), a B-type response regulator with no ortholog in Arabidopsis [15]; and *Hd3a*, a phosphatidylethanolamine-binding protein and the ortholog of Arabidopsis FT [16].

Using a core collection of 64 rice cultivars that represent the genetic diversity of 332 accessions from around the world, Takahashi et al., demonstrated that the variation in *Hd1*, *Ehd1*, and *Hd3a* contributed to the flowering time diversity in cultivated rice [17]. They suggested that Hd1 proteins, *Hd3a* promoters, and *Ehd1* expression levels together contributed to the diversity of flowering time. Later on, using 60 accessions of *Oryza sativa* and 38 accessions of *Oryza rufipogon*, this group showed that *Hd3* was highly conserved over time, whereas *Hd1* underwent human selection. Thus, they suggested *Hd1* was a possible target for domestication in the heading-date trait [7]. The flowering strategy of US weedy rice was studied recently and again *Hd1* was found important, although the *Hd1* haplotype alone could not fully explain the differences in flowering phenotype [18].

In temperate regions such as Japan, modern rice varieties such as Nipponbare are transplanted into the field in early June and harvested in late fall, for only one cropping season each year, and the yield is reasonable. In subtropical regions like Taiwan, modern local rice varieties such as Tainung 67 (TNG67) may be planted twice a year, with the first cropping season from February to June and the second from July to November. That is, seeds harvested from the first cropping season may germinate and be planted the next month. However, if Nipponbare were planted in Taiwan, it would flower very early or very late, depending on the transplanting time, so grain yield would be affected. However, how Taichung 65 (TC65, abbreviated as T65 in some papers), a variety that may be grown two times a year in Taiwan, lost its photoperiod sensitivity even though both of its parental lines are sensitive to photoperiod, has been a puzzle for a long time.

In the present study, we used SNPs obtained from whole genome sequencing to examine how TC65 gained different photoperiod sensitivity. We found that introgression must have occurred decades ago from upland rice accessions grown nearby and that TC65 had four accessions in its pedigree. This is a good example of introgression playing important roles during rice domestication.

2. Materials and methods

2.1. Plant materials and growth conditions

Rice TC65 seeds were obtained from Professor Motoyuki Ashikari (Nagoya University, Japan). The seeds of TNG67, Kameji, Shinriki and all Taiwan landraces as well as modern varieties were obtained from the Plant Germplasm Division, Taiwan Agriculture Research Institute, Taiwan. The breeders' seeds harvested decades ago were used for the modern varieties.

For DNA extraction, the plants of single seed descent were cultivated until tillering stage in an Academia Sinica greenhouse under natural light. Healthy leaves without insect damage from one plant were harvested, frozen under liquid nitrogen and stored at −80 °C. For heading date and agronomic trait analysis, the plants were cultivated in the experimental paddy field under natural light at agricultural research institutes or stations, including Academia Sinica (Taipei, 2012), Taiwan Agriculture Research Institute (Taipei, 1961, 1962), Taiwan Agriculture Research Institute (Taichung, 2012, 2014), and Tainan District Agricultural Research and Extension Station (Tainan, 2014). Twenty plants per accession, with two duplicates in Tainan, were transplanted. Heading date was defined as the time when the first panicle appeared from the node in half of these plants.

2.2. Genomic DNA extraction, PCR analysis, and sequencing

Genomic DNA was extracted from leaves by using a DNeasy Plant Mini Kit (Qiagen). After quality assessment, the genomic DNA was randomly fragmented and size-fractionated. DNA fragments with desired lengths were gel-purified. For whole-genome resequencing, paired-end libraries with 450–500-bp inserts were constructed and sequenced on a GA2 or HiSeq2000 system (Illumina). Sequence data were deposited in the NCBI Sequence Read Archive. The functional SNP (FNP) for *Hd1*, *Ehd1*, *qSH1* and *SH4* were amplified by PCR followed by capillary sequencing analysis. Primer sequences are in Table S1.

2.3. SNP and indel calling

Adaptor sequences, low-quality bases and reads <20 bp long were discarded. The trimmed paired reads were then aligned to the reference rice Nipponbare genome sequence (IRGSP v1.0). SAMtools and VCFtools [19] were used to manipulate and transform the SAM and variant call format (VCF) [19] file format. To detect SNPs and small indels, we used the command lines in the section "EXAMPLES" in the SAMtools manual without any restriction on depth or mapping quality. The information on SNP and small indels was recorded in VCF files, compared by "vcf-isec" to classify sample-specific or intersection variants, then imported into Integrative Genomics Viewer (IGV) [20,21] to show alleles or genotypes.

All split VCF files were filtered and checked by visualization, then the regional files were output in BED format (.bed). We used snapshot image files from IGV [21] for variant events on the NGS mapped images. Locations of SNPs were then manually validated. The curated SNPs along the 12 chromosomes were plotted by using BasicChromosome module of Biopython [22]. SNPeff [23] was used to calculate the location of each SNP in the genome.

2.4. Estimation of heterozygosity

The VCF files were used to estimate heterozygosity, then the common SNPs for the 5 accessions were filtered out. To screen for heterozygous SNPs, 10 kb was used as a window size. If the number of heterozygous SNPs was greater than two times that of the homozygous SNPs, the block was considered heterozygous. In total, 37,440 blocks occur in the rice genome, and the percentage heterozygosity was then calculated.

2.5. Measurement of agronomic traits

The color of apiculus, presence of awn, flag leaf length and the plant types of the accessions were monitored by visualization. Shattering was measured as the percentage of spikelets that fell from each panicle. Near-infrared spectroscopy was used to estimate seed protein, amylose, moisture content and brown rice score (NIRT Grain Tester AN-900, Kett Electric Lab, Tokyo). The resistance to seedling blast was monitored as R (resistant), M (moderate), and S (susceptible). The resistance to sheath blight was screened by degree of severity of sheath blight (H): $H = 3n_1 + 2n_2 + 1n_3 + 0n_4/3N \times 100$, where $N = n_1 + n_2 + n_3 + n_4$ (n_1 = number of leaves with symptoms in the flag leaf and second, third and fourth leaf blade or sheath; n_2 = number of leaves with symptoms in the second, third and fourth leaf blade or sheath; n_3 = number of leaves with symptoms in the third and fourth leaf blade or sheath and any leaf underneath; and n_4 = number of healthy leaves [24]).