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**Identification of the gene for thermosensitivity associated with heading in
Hokkaido rice varieties**

(北海道イネ品種に見られる出穂に関連した温度感受性遺伝子の同定)

A summary of the thesis

Required for the partial fulfillment of the
the degree of Doctor of Philosophy (PhD)

(Laboratory of Plant Breeding)

北海道大学 大学院農学院

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Background

Rice (*Oryza sativa* L.) is a fundamental global food source, feeding over half the world's population. Its cultivation spans diverse climates, from tropical regions to high-latitude areas like Hokkaido, Japan (over 40°N). The ability of rice to adapt to these different environments is largely determined by its heading date, a critical agronomic trait that controls the timing of flowering. As a short-day plant, rice flowering is sensitive to day length, but significant natural variation in this trait exists among cultivars.

Hokkaido, one of the northernmost rice-farming regions in the world, presents a unique challenge for rice cultivation due to its short growing season and long summer day lengths. A century ago, producing rice in this climate was extremely difficult. However, through a hundred years of intensive artificial selection and strategic breeding programs, Hokkaido has been transformed into one of Japan's premier rice-producing areas. This success was achieved by developing cultivars specifically adapted to the northern climate.

Hokkaido rice varieties are characterized by early maturity, a short time to heading (Days to Heading), and very low photoperiod sensitivity, allowing them to flower and mature quickly despite the long summer days. This adaptation is the result of focused selection for genetic traits that optimize growth and yield in a compressed timeframe. While the genetic diversity of Hokkaido rice is part of a narrower subset of global rice variation, it represents a valuable and distinct regional gene pool. Studies have identified four specific genetic loci that differentiate the heading date control in Hokkaido varieties from those grown on Japan's main island of Honshu.

Despite this progress, a significant knowledge gap remains. The specific genes responsible for the characteristic earliness in Hokkaido cultivars are still largely unexplored. Understanding the precise genetic mechanisms that govern this crucial adaptation is essential for future rice improvement. Investigating the genetic properties of heading traits in Hokkaido's unique varieties offers a valuable opportunity to identify novel genes. This knowledge can, in turn, contribute to the development of new and more effective breeding strategies for improving rice adaptation and productivity in high-latitude agricultural systems worldwide.

Cryptic heading variation among rice varieties in Hokkaido is responsible for regional genetic diversity

(Chapter 2)

Introduction

Studying the genetic basis for local adaptation is challenging because the contributions of region-specific genetic variants are often small and difficult to distinguish from environmental effects. However, these subtle variations within Locally Adapted Varieties (LAVs) are crucial for fine-tuning traits and provide valuable options for regional breeding programs. Rice (*Oryza sativa* L.) offers a clear example of regional adaptation. Although native to the tropics, it has been successfully cultivated at high latitudes (above 40°N) like Hokkaido, Japan, by acquiring key traits (Fujino and Shirasawa, 2022). While cold tolerance is one such trait, its genetic basis is complex. A more clearly understood adaptation is early heading (early flowering), which allows the plant to complete its life cycle in a short growing season. This trait is primarily caused by a loss of photoperiod sensitivity, which has been linked to nonfunctional alleles in major genes such as *Ghd7*, *OsPRR37*, *Hd1*, and *DTH8* (Fujino et al., 2019a; Shinada et al., 2014). This study aims to characterize the variation in Days to Heading (DTH) among Hokkaido's LAVs. The goal is to reveal the contributions of these small-effect genes, which fine-tune this critical adaptive trait. This research is significant because it focuses on the subtle genetic landscape relevant to local breeding, a type of variation that is often overlooked in studies that rely on crosses between genetically distant lines where large-effect genes dominate the outcomes.

Methodology

This study measured the Days to Heading (DTH) for 79 Hokkaido rice varieties at the Kamikawa Agricultural Experimental Station. The DTH data was collected over seven years (from 2013-2018 and 2020-2023, excluding 2017 and 2023 as statistical outliers), with heading dates recorded when 50% of tillers had flowered on 20 plants per variety. Genotypes for four key photoperiod-related loci (*Ghd7*, *OsPRR37*, *Hd1*, and *DTH8*) were obtained from a previous dataset. The statistical association between these genotypes and the measured DTH was then analyzed using one-way ANOVA, t-tests, and the Tukey-Kramer test with Prism and Excel software.

Results

The genetic basis for early heading was investigated by examining the alleles of four known photoperiod-related genes: *Ghd7*, *OsPRR37*, *Hd1*, and *DTH8*. The results clearly showed that early heading in Hokkaido varieties is predominantly controlled by the two major genes that suppress flowering. Major Effect Genes: The vast majority of the 70 genotyped varieties carried nonfunctional alleles for *Ghd7* (86%) and *OsPRR37* (93%). The presence of a nonfunctional allele at these loci had a powerful effect, accelerating flowering by an average of 8.6 days (*Ghd7*) and 9.7 days (*OsPRR37*), respectively. Minor Effect Genes: In contrast, the effects of *Hd1* and *DTH8* were much smaller. A functional *Hd1* allele hastened flowering by only 3.0 days, while *DTH8* had a negligible effect of 0.3 days. The combined effect of *Ghd7* and *OsPRR37* was the most critical factor. Varieties that had nonfunctional alleles for both of these genes showed consistently early and similar heading dates (averaging 56.3 days). In this genetic context, the smaller effects of *Hd1* and *DTH8* were largely masked by the larger environmental variation (Fig. 1).

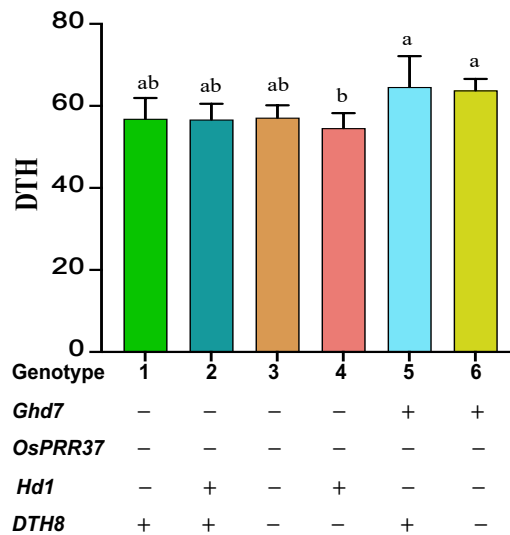


Fig.1: Effect of the combined genotype at *Hd1*, *Ghd7*, *OsPRR37*, and *DTH8* on DTH.

Discussion:

Rice adaptation in Hokkaido is a two-tiered process driven by a distinct genetic strategy to cope with environmental pressures. The fundamental requirement for early flowering in the region's short, cool growing season is primarily established by the widespread loss-of-function of two major-effect genes, *Ghd7* and *OsPRR37*, which prevents flowering delays under long summer days

(Ebana et al., 2010). Within this essential adaptive framework, minor-effect genes like *Hd1* and *DTH8*, along with other unknown genetic factors, provide subtle variations that fine-tune the flowering time (Hori et al., 2013; Zhang et al., 2015). This critical balance between strong genes for core survival and weaker genes that maintain diversity allows rice to thrive at its cultivation limit, while still offering breeders the genetic flexibility to develop improved local varieties that must also contend with significant year-to-year environmental fluctuations.

Conclusion

Genes such as *Ghd7*, *OsPRR37*, *Hd1*, and *DTH8* determine the heading time of Hokkaido's locally adapted rice varieties (LAVs). Most Hokkaido varieties contain unfunctional alleles for *Ghd7* and *OsPRR37*, indicating these are the main factors responsible for early heading. In contrast, the functional and unfunctional alleles in *Hd1* and *DTH8* are shared by Hokkaido varieties, suggesting that these genes are responsible for varying degrees of heading earliness. Despite the low level of phenotypic variation in Hokkaido LAVs, heading time may be controlled by variable genes with small effects. Unidentified genetic variations may customize rice varieties to meet particular local demands.

Identification of the gene for thermosensitivity associated with heading in Hokkaido rice varieties

(Chapter 3)

Introduction

This study employed Quantitative Trait Locus (QTL) mapping to identify the genetic basis of heading earliness in Hokkaido rice. Building on previous research that used a Recombinant Inbred Line (RIL) population from a cross between the Hokkaido lines 'A58' and 'Kitaake', five novel QTLs for Days to Heading (DTH) were identified (Koide et al., 2019). The most significant of these, qFDTH4, was located on chromosome 4. The primary goal of the current research is to perform fine mapping on this specific qFDTH4 region. This will narrow down the genomic interval to precisely pinpoint the candidate gene or genes responsible for controlling heading earliness,

offering a deeper insight into the molecular mechanisms behind this critical adaptive trait for high-latitude rice cultivation.

Methodology

To identify a gene for heading date by fine mapping a specific region on rice chromosome 4. A large mapping population, derived from a cross between the Hokkaido rice lines 'A58' and 'Kitaake', was grown over three consecutive years (2022-2024), and their individual heading dates were meticulously recorded. To uncover the underlying genetic differences, the parental lines underwent whole-genome sequencing, and the resulting data were analyzed to identify polymorphisms like SNPs and InDels. These variations were then used to design PCR-based molecular markers, which, along with Sanger sequencing, were used to genotype the entire mapping population.

Results

This study successfully identified the gene underlying qFDTH4, a major Quantitative Trait Locus (QTL) on rice chromosome 4 that controls heading date. After initially confirming the QTL's significant effect (a 2.1-day difference in heading time) in a panel of Hokkaido varieties, a large fine mapping population was developed from a cross between the parental lines 'A58' and 'Kitaake' (Table 1). Systematic genotyping of thousands of individuals over three years successfully narrowed the location of the gene to a 90.43 kb region. Within this interval, the gene was identified as the prime candidate due to a specific polymorphism present in the earlier-heading 'Kitaake' parent and its high expression in young floral tissues. This conclusion was validated through a haplotype analysis of 38 Hokkaido varieties, which confirmed that the presence of this polymorphism is significantly associated with a 2.2-day acceleration in heading date, providing strong evidence for its role in fine-tuning flowering time in high-latitude rice.

Table 1: Heading days distribution of several selected lines progeny from F₆ populations

No.	Line No.	No. of Individuals	Average DTH	Most frequent DTH	Maximum DTH	Minimum DTH
1	128-2	114	82.3	85	87	69
2	128-3	120	76.6	82	87	63
3	135-3	114	70.4	67	84	63
4	135-4	114	71.1	71	83	63
5	135-5	120	71.2	67	85	63

Heading dates were evaluated in the paddy field of Hokkaido University every day, and the first appearance of the panicle from seed sowing was considered for DTH calculation.

Discussion

Small-effect gene that fine-tunes the heading date in Hokkaido rice by exploiting hidden genetic diversity between two phenotypically similar parental varieties, 'A58' and 'Kitaake'. The appearance of a wide range of heading dates in their progeny, known as transgressive segregation (Koide et al. 2019), enabled the mapping of a new Quantitative Trait Locus (QTL), qFDTH4, which was found to have a small but consistent and agriculturally significant effect, altering the heading date by more than two days. This research reveals that the gene acts as an independent regulator that works alongside major heading date genes, providing a crucial mechanism for fine-tuning flowering time and optimizing adaptation in Hokkaido's short growing season.

Conclusion

This discovery, made possible through the analysis of transgressive segregation in a cross between phenotypically similar parents, reveals the hidden genetic diversity controlling the early heading

trait. Acting independently of major known flowering time regulators, serves as a crucial component for fine-tuning this critical agronomic trait. This finding not only adds a new piece to the complex polygenic network governing heading date but also underscores the importance of studying locally adapted varieties to uncover genes vital for regional agricultural adaptation.

Thermo-sensitivity of heading date found in Hokkaido rice varieties influenced by the novel gene affects heading synchrony and premature heading

(Chapter 4)

Introduction

Hokkaido, where early heading is essential to avoid yield loss from autumn cold. As climate change alters growing conditions, understanding the genetic control of this trait is crucial for developing resilient varieties (Fujino and Ikegaya, 2020). Unlike tropical rice, which relies on major photoperiod genes, Hokkaido cultivars have adapted to their short, long-day season primarily through the accumulation of multiple small-effect Quantitative Trait Loci (QTLs) that fine-tune their early flowering. Therefore, this study aims to identify and characterize these specific small-effect genes through fine mapping. The findings will provide a deeper molecular understanding of heading earliness and will be instrumental for marker-assisted selection in breeding programs, ultimately contributing to the development of stable, high-yielding cultivars that ensure food security in temperate regions.

Methodology

The methodology involved gene expression analysis, environmental response observation, and functional gene validation using CRISPR/Cas9. Total RNA was extracted from the young panicles of parental lines ‘A58’ and ‘Kitaake’ for comparative RNA-seq analysis, while early- and late-heading progeny were grown under different temperature and day-length conditions to observe the effects on meristem development and heading date. To functionally test the candidate gene, a CRISPR/Cas9 system was constructed to introduce targeted mutations, as well as the known heading genes *Ghd7* and *Hd1*. This was achieved by using *Rhizobium radiobacter*-mediated

transformation to deliver the CRISPR construct into ‘Nipponbare’ rice calli, and the successful genetic edits in the regenerated plants were subsequently confirmed by Sanger sequencing.

Results

This study's results reveal that the candidate gene qFDTH4 is an essential regulator of rice development, whose function is modulated by an Indel in the ‘Kitaake’ allele. RNA-seq analysis confirmed this deletion causes a significant molecular disruption, resulting in an altered transcript structure and a threefold reduction in gene expression compared to the ‘A58’ allele. This altered expression has distinct phenotypic consequences, conferring increased temperature sensitivity at the panicle initiation stage and causing both an earlier first heading and a longer heading synchrony (the time between subsequent panicles) (Fig. 2). The gene's fundamental importance was ultimately demonstrated by a CRISPR/Cas9 experiment; the inability to recover any viable knockout mutants, despite successful transformation, strongly indicates that a complete loss of qFDTH4 function is lethal, highlighting its essential role in plant survival.

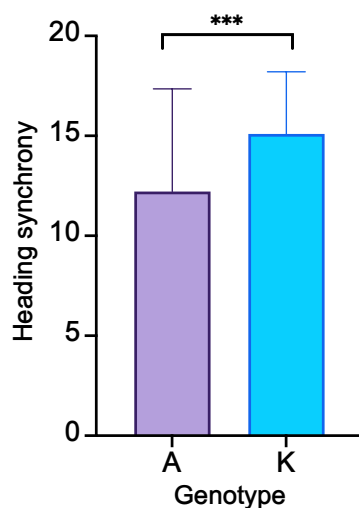


Fig. 2 : Heading synchrony variation of homozygous A58 (A) type and Kitaake (K) type allele

Discussion

The qFDTH4 is a highly conserved and essential gene whose function is modulated by a key deletion in the ‘Kitaake’ allele. This deletion causes transcriptional disruption, resulting in an

altered transcript and reduction in gene expression. This molecular change has significant functional consequences, conferring increased thermosensitivity that accelerates panicle development in response to warmer temperatures. Phenotypically, this manifests as a significantly earlier first heading and a longer heading synchrony (Takeda, 1986). Haplotype analysis further supports the gene's role in local adaptation, showing that the allele containing this deletion is prevalent in temperate rice varieties at high latitudes, confirming its importance in fine-tuning flowering time for success in short growing seasons.

Conclusion

The Kitaake type genotypes exhibited significantly longer heading synchrony than the A58 type genotypes. If the A58 likely contains wild type allele, the gene in Kitaake may function to repress ectopic initiation of the panicle by the high temperature. The knockout individuals of the candidate genes couldn't survive that may cause the lethal action of the null of the gene. It is hypothesized that cells with successful edits were non-viable and could not regenerate into whole plants, thus preventing the recovery of any mutants.

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