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## Research Paper

# Molecular and genealogical analysis of grain dormancy in Japanese wheat varieties, with specific focus on *MOTHER OF FT AND TFL1* on chromosome 3A

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In the wheat (*Triticum aestivum* L.) cultivar ‘Zenkoujikomugi’, a single nucleotide polymorphism (SNP) in the promoter of *MOTHER OF FT AND TFL1* on chromosome 3A (*MFT-3A*) causes an increase in the level of gene expression, resulting in strong grain dormancy. We used a DNA marker to detect the ‘Zenkoujikomugi’-type (Zen-type) SNP and examined the genotype of *MFT-3A* in Japanese wheat varieties, and we found that 169 of 324 varieties carry the Zen-type SNP. In Japanese commercial varieties, the frequency of the Zen-type SNP was remarkably high in the southern part of Japan, but low in the northern part. To examine the relationship between *MFT-3A* genotype and grain dormancy, we performed a germination assay in three wheat-growing seasons. On average, the varieties carrying the Zen-type SNP showed stronger grain dormancy than the varieties carrying the non-Zen-type SNP. Among commercial cultivars, ‘Iwainodaichi’ (Kyushu), ‘Junreikomugi’ (Kinki-Chugoku-Shikoku), ‘Kinuhime’ (Kanto-Tokai), ‘Nebarigoshi’ (Tohoku-Hokuriku), and ‘Kitamoe’ (Hokkaido) showed the strongest grain dormancy in each geographical group, and all these varieties, except for ‘Kitamoe’, were found to carry the Zen-type SNP. In recent years, the number of varieties carrying the Zen-type SNP has increased in the Tohoku-Hokuriku region, but not in the Hokkaido region.

**Key Words:** *Triticum aestivum* L., grain dormancy, pre-harvest sprouting, *MFT*, genealogical pedigree.

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## Introduction

Germination of wheat grains on the mother plant before harvest is called pre-harvest sprouting (PHS). PHS decreases the quality of the end product, coupled with lower grain yield, and causes economic losses (Bewley and Black 1994). Therefore, PHS tolerance has been an important breeding objective in wheat-breeding programs. The most suitable way to improve PHS tolerance is to select varieties with higher grain dormancy at harvest. However, grain dormancy is strongly influenced by environmental conditions, such as temperature and humidity (rainfall and aridity), during grain development (King 1993, Lunn *et al.* 2002, Mares *et al.* 2009). Thus, unstable evaluation of grain dormancy makes it difficult to select PHS-tolerant varieties in the field.

The wheat acreage in Japan is about 210,200 ha and its production reached nearly 811,700 tons in 2012, while the wheat consumption per year is 7,167,000 tons. Annual production of wheat covers only 11% of Japanese consump-

tion. One of the factors that limit wheat production in Japan is the low quality due to PHS. Wheat is mainly cultivated in the Hokkaido, Kanto-Tokai, and Kyushu regions. In southern parts of Japan, including the Kanto-Tokai and Kyushu regions, the harvest maturity of wheat coincides with the beginning of the monsoon season. Hokkaido, which is the most productive region in Japan, has a cool humid summer every few years. These weather conditions easily lead to sprouting if rain comes before harvest. Although the newly bred commercial varieties in the Hokkaido region were improved for PHS tolerance (Yanagisawa *et al.* 2005), varieties with still higher PHS tolerance are needed. In Hokkaido, wheat production in 2009 (400,100 t) was 26% lower than that in 2008 (541,500 t) because of the occurrence of PHS under conditions of continuous rain and cool weather just before the harvest (Nishio *et al.* 2011).

The history of wheat breeding in Japan has been described in detail by Hoshino and Seko (1996). Since 1934, through the Japanese wheat-breeding programs, many Japanese and introduced foreign varieties have been subjected to germination assays and/or sprouting tests to find the source of PHS tolerance (Akihama 1936, Fukunaga *et al.* 1987, Oda and Seko 1992). Japanese varieties, especially the

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Communicated by H. Tsujimoto

Received August 25, 2014. Accepted October 20, 2014.

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varieties that originated or bred in southern parts of Japan, are generally more dormant than foreign varieties. Hoshino *et al.* (1989) reported that ‘Zenkoujikomugi’ has the highest dormancy in the genealogical pedigree of Japanese wheat varieties that they used. Although many breeders have tried to establish a new variety with stronger grain dormancy than ‘Zenkoujikomugi’, no such variety has been developed from crosses between ‘Zenkoujikomugi’ and other varieties with strong grain dormancy. It is presumed that the key gene regulating grain dormancy is identical in ‘Zenkoujikomugi’ and the varieties used by breeders. The varieties developed in southern parts of Japan might have potentially acquired PHS tolerance during the long history of wheat cultivation under the moist conditions; however, the crucial factor that affects PHS tolerance has not been identified.

Many analyses of quantitative trait loci (QTLs) have been conducted for PHS tolerance and grain dormancy in wheat, and a number of QTLs have been found on wheat chromosomes (Anderson *et al.* 1993, Flintham *et al.* 2002, Groos *et al.* 2002, Kato *et al.* 2001, Kulwal *et al.* 2012, Mori *et al.* 2005, Munkvold *et al.* 2009, Osa *et al.* 2003, Torada *et al.* 2008). A major QTL, *QPhs.ocs-3A.1*, was mapped to the terminal region of the short arm of chromosome 3A by using recombinant inbred lines derived from a cross between the highly dormant variety ‘Zenkoujikomugi’ and the less dormant variety ‘Chinese Spring’ (Mori *et al.* 2005). In recent years, a wheat homologue of *MOTHER OF FT AND TFL1* (*MFT*) underlying *QPhs.ocs-3A.1* was identified, and a cleaved amplified polymorphic sequence (CAPS) marker was developed to detect a single nucleotide polymorphism (SNP) in the promoter of *MFT* on chromosome 3A (*MFT-3A*) (Nakamura *et al.* 2011). They showed that a nucleotide substitution in *MFT-3A* of ‘Zenkoujikomugi’ increases the transcriptional level of *MFT*, resulting in strong grain dormancy. Although the ‘Zenkoujikomugi’-type (Zen-type) SNP in the promoter of *MFT-3A* is thought to play an important role in the control of grain dormancy in wheat, it remains unknown whether the Zen-type SNP is common in other Japanese wheat varieties.

The objective of our study was to identify varieties carrying the Zen-type SNP in the promoter of *MFT-3A* and to lay the foundation for marker-assisted breeding aimed at improving PHS tolerance in wheat. We performed CAPS marker analysis to identify the genotype of *MFT-3A* in Japanese wheat varieties. The level of grain dormancy in each variety was evaluated using germination assays on freshly harvested grains, and the relationship between *MFT-3A* genotype and grain dormancy was discussed. The genealogical pedigree of newly developed varieties with strong grain dormancy was also discussed.

## Materials and Methods

### Plant materials

A total of 324 wheat varieties (Supplemental Table 1), including 183 commercial varieties released in Japan (174

Norin varieties and 9 varieties without Norin number), 62 Japanese landraces, 39 breeding lines, and 40 foreign varieties introduced for breeding, were used in the present study. Norin varieties have been registered with the Ministry of Agriculture, Forestry and Fisheries, and have also been assigned a name in addition to the Norin number, starting from Norin 76 (‘Yuyakekomugi’). The geographical origins are summarized in Supplemental Table 1.

### CAPS marker analysis

For DNA extraction, all wheat genotypes were grown in a growth chamber maintained at 20°C with a natural photoperiod. Genomic DNA was extracted from 2-week-old seedlings by using a modified CTAB method (Murray and Thompson 1980). Wheat varieties were genotyped by using a CAPS marker for the SNP in the promoter region of *MFT-3A*, according to the method described by Nakamura *et al.* (2011). PCR was performed with a gene-specific primer pair (CS3A06Proseq-F3: 5'-GTAGCGGGTGAAATCTGCAT-3' and CS3A06Proseq-R5: 5'-GGGACGTACGAGGGTGTAGA-3') that was designed to amplify the region around the SNP in the promoter sequence of *MFT-3A* and Ex Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. The PCR conditions were as follows: 30 s denaturation at 98°C, followed by 40 cycles of 10 s at 98°C, 30 s at 60°C, and 1 min at 72°C. The amplified fragments of about 800 bp were treated with *Clal* restriction enzyme (Takara Bio Inc.), and the fragment size was analyzed using polyacrylamide gel electrophoresis. The amplified fragments with the Zen-type SNP cannot be cut by *Clal* because of nucleotide substitution. The amplified fragments with the ‘Chinese spring’-type SNP were cut by *Clal*, producing two fragments of approximately 400 bp. To achieve the required separation efficiency, a high-efficiency genome scanning system (Kawasaki and Murakami 2000) was used.

### Evaluation of grain dormancy

A total of 267 varieties (160 commercial varieties, 29 Japanese landraces, 39 breeding lines, and 39 foreign varieties introduced for breeding) were sown and grown in a field at NARO Agricultural Research Center (36°01' N, 140°06' E) in the Kanto region and subjected to a germination assay for 2004/2005, 2005/2006, and 2006/2007 wheat-growing seasons. Each experimental plot consisted of a single 1.0-m-long row, and the planting distance was 70 cm between rows and 8.5 cm between plants. For germination assay, spikes were harvested from the field at physiological maturity, as estimated by the loss of green color from the glumes, and were dried at room temperature for 10 days. Dried spikes were gently threshed, and the collected grains were stored at 4°C before use. Thirty whole grains were placed in a petri dish (9 cm diameter) containing two Whatman No. 2 filter papers (8.5 cm diameter) and 6 ml of water, with three replicates. Dishes were placed in a growth cabinet at 20°C. Germinated grains (germination was defined as pericarp rupture over the embryo) were counted and removed from

the dishes every 24 h for 7 days. Germination was represented by the index GI. GI was calculated with the maximum weight given to the grains that germinated first and less weight to those that germinated later.

$$GI = [(7 \times n_1 + 6 \times n_2 + 5 \times n_3 + 4 \times n_4 + 3 \times n_5 + 2 \times n_6 + 1 \times n_7) / (7 \times \text{total grains})] \times 100$$

where  $n_1, n_2, \dots, n_7$  is the number of grains germinated on the first, second, and subsequent days until the seventh day, respectively. The average and standard error (SE) were calculated. The GI values of the varieties carrying the Zen-type SNP and non-Zen-type SNP were compared using a *t*-test.

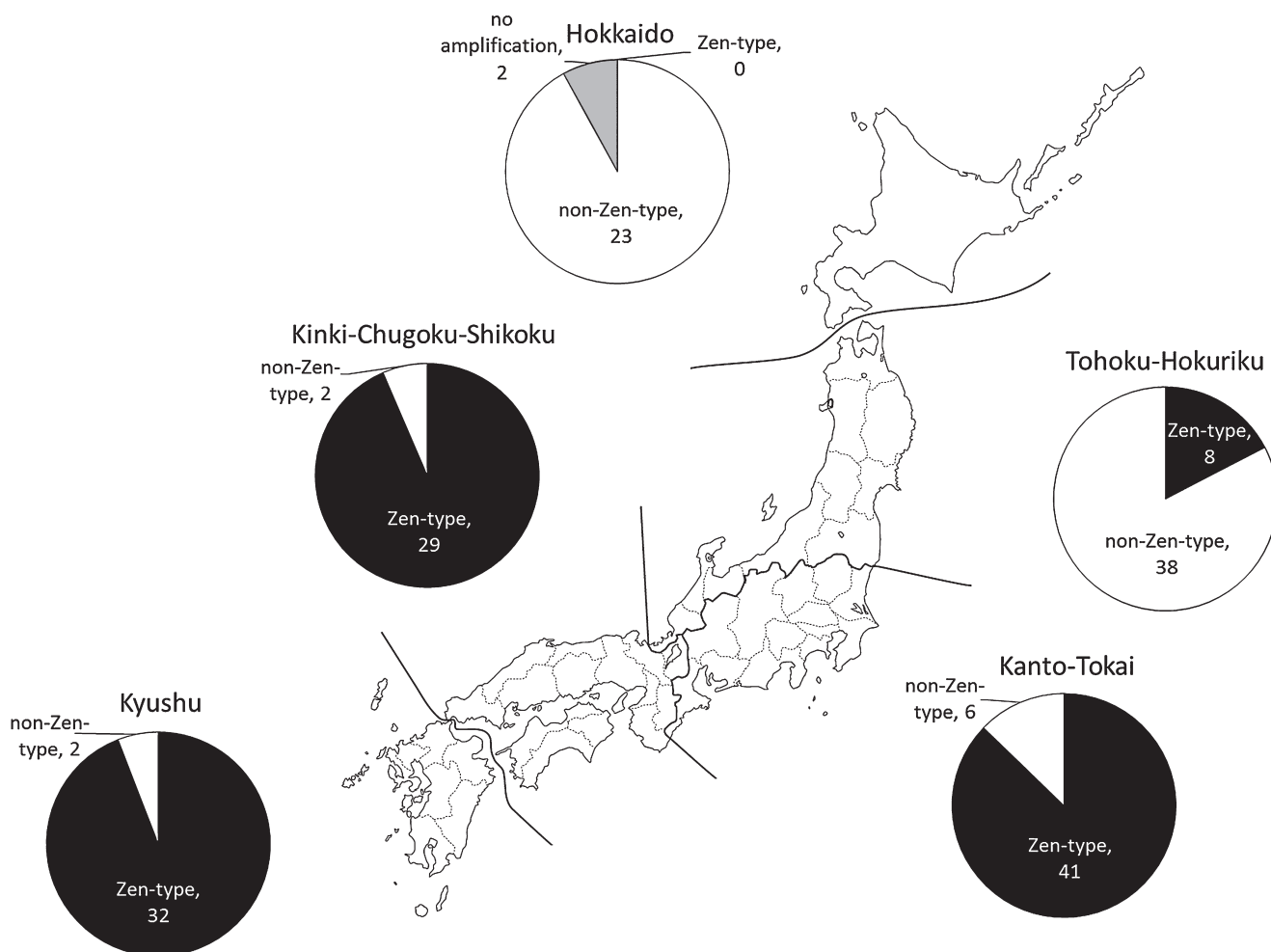
To evaluate the grain dormancy of ‘Kitahonami’, the commercial varieties bred in the Hokkaido region (‘Horoshirikomugi’, ‘Takunekomugi’, ‘Chihokukomugi’, ‘Hokushin’, ‘Kitamoe’, and ‘Kitahonami’) were sown in a field at NARO Agricultural Research Center and subjected to a germination assay for 2006/2007 and 2007/2008 wheat-growing seasons. These six varieties were sown separately from the

varieties in the large-scale evaluation of grain dormancy for the 2006/2007 wheat-growing season. Germination was represented by GI.

## Results and Discussion

### *MFT-3A* genotyping in Japanese wheat varieties

The genotype of *MFT-3A* in Japanese wheat varieties was examined using PCR with the CAPS marker developed by Nakamura *et al.* (2011). A total of 324 wheat varieties, consisting of commercial varieties, Japanese landraces, breeding lines, and foreign varieties introduced for breeding, were subjected to the analysis. The DNA fragment of the expected size (about 800 bp) was amplified in 320 varieties. We could not amplify the DNA fragment from two commercial varieties (‘Horoshirikomugi’ and ‘Chihokukomugi’) and two breeding lines (‘Kitakei 320’ and ‘Kitakei 497’) with this CAPS marker (**Supplemental Table 1**). These varieties were developed in the Hokkaido region. Detailed analysis of the *MFT-3A* sequence of ‘Chihokukomugi’



**Fig. 1.** Genotype distribution of the Zen-type SNP and non-Zen-type SNP in Japanese commercial varieties. Solid and open parts of the circular chart indicate the proportions of wheat varieties carrying the Zen-type SNP and non-Zen-type SNP, respectively. Gray parts of the circular chart indicate the proportion of wheat varieties with no amplification with the CAPS marker. Number of varieties is indicated.

**Table 1.** Varieties of wheat with lower average GI values than ‘Zenkoujikomugi’. All the varieties carried the Zen-type SNP in the promoter of *MFT-3A*

Name	Origin	Germination Index				SE
		2004/2005	2005/2006	2006/2007	Average	
Igachikugo Oregon	Kanto-Tokai	9.7	10.7	16.2	12.2	2.0
Aki 9	Kyushu	25.2	16.0	3.6	14.9	6.3
Junreikomugi	Kinki-Chugoku-Shikoku	11.0	23.8	21.2	18.7	3.9
Kinuhime	Kanto-Tokai	31.9	16.4	12.6	20.3	5.9
Chugoku 81	Kinki-Chugoku-Shikoku	11.4	33.8	16.0	20.4	6.8
Shinrikikomugi	Kinki-Chugoku-Shikoku	32.4	12.1	25.2	23.3	5.9
Zenkoujikomugi	Kanto-Tokai	5.7	36.2	29.3	23.7	9.2

revealed that ‘Chihokukomugi’ has an insertion in *MFT-3A* (Nakamura *et al.* unpublished data). Further investigation of *MFT-3A* genotype in wheat varieties will soon be reported. Sequence variation of *MFT* in wheat has also been reported by Lei *et al.* (2013) and Liu *et al.* (2013).

On the basis of the fragment pattern after *Cla*I digestion, 320 varieties were classified into two types: one carrying the Zen-type SNP (cannot be cut by *Cla*I) and the other carrying the non-Zen-type SNP (can be cut by *Cla*I). In our experiments, 110 of 181 commercial varieties, 34 of 62 Japanese landraces, 23 of 37 breeding lines, and 2 of 40 foreign varieties were found to carry the Zen-type SNP in the promoter of *MFT-3A* (Supplemental Table 1). Two foreign varieties carrying the Zen-type SNP were ‘Joshuu’ and ‘Shikan’, both originated in China.

### Geographic distribution of the Zen-type SNP in Japanese commercial varieties

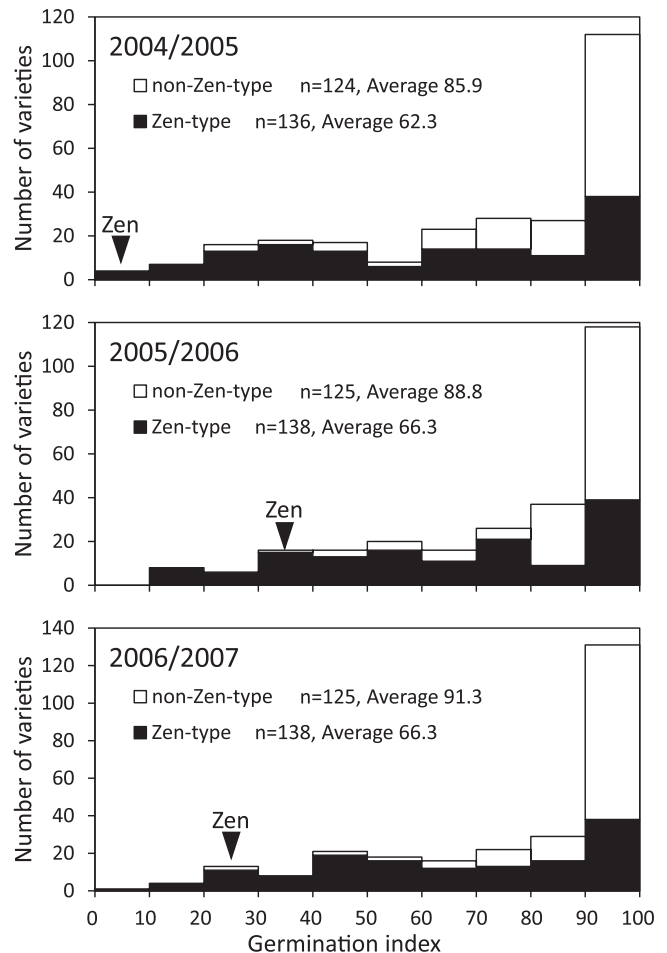
The frequency of the Zen-type SNP was different among the geographical groups of 183 commercial varieties (Fig. 1). The varieties carrying the Zen-type SNP were mainly developed in the southern parts of Japan. In the Kyushu, Kinki-Chugoku-Shikoku, and Kanto-Tokai regions, 32 of 34 varieties (94.1%), 29 of 31 varieties (93.5%), and 41 of 47 varieties (87.2%), respectively, were found to carry the Zen-type SNP. In contrast, few varieties carrying the Zen-type SNP were developed in the northern parts of Japan. In the Tohoku-Hokuriku region, only 8 of 46 varieties (17.4%) were found to carry the Zen-type SNP. In the Hokkaido region, none of the 25 commercial varieties carried the Zen-type SNP.

During wheat harvest in Japan, rain and high humidity are frequent. In such conditions, a variety carrying the Zen-type SNP may be preferred because of its potentially strong grain dormancy. Although strong grain dormancy prevents PHS at harvest, it hinders rapid and uniform germination at the beginning of the next growing season. In the southern parts of Japan, dormancy decay during dry storage may be sufficient to minimize the non-uniform germination at sowing.

### Relationship between *MFT-3A* genotype and grain dormancy in Japanese wheat varieties

Grain dormancy in Japanese wheat varieties was evaluated by GI calculated from the data obtained from germina-

tion assays for three wheat-growing seasons (Supplemental Table 1). A total of 267 varieties were subjected to the germination assay, and three varieties (‘Asakazekomugi’, ‘Fukuhokomugi’, and ‘Furutsudaruma’) were not grown in 2004/2005 wheat growing season. The average GI values of ‘Igachikugo Oregon’, ‘Aki 9’, ‘Junreikomugi’, ‘Kinuhime’, ‘Chugoku 81’, and ‘Shinrikikomugi’ were lower than that of



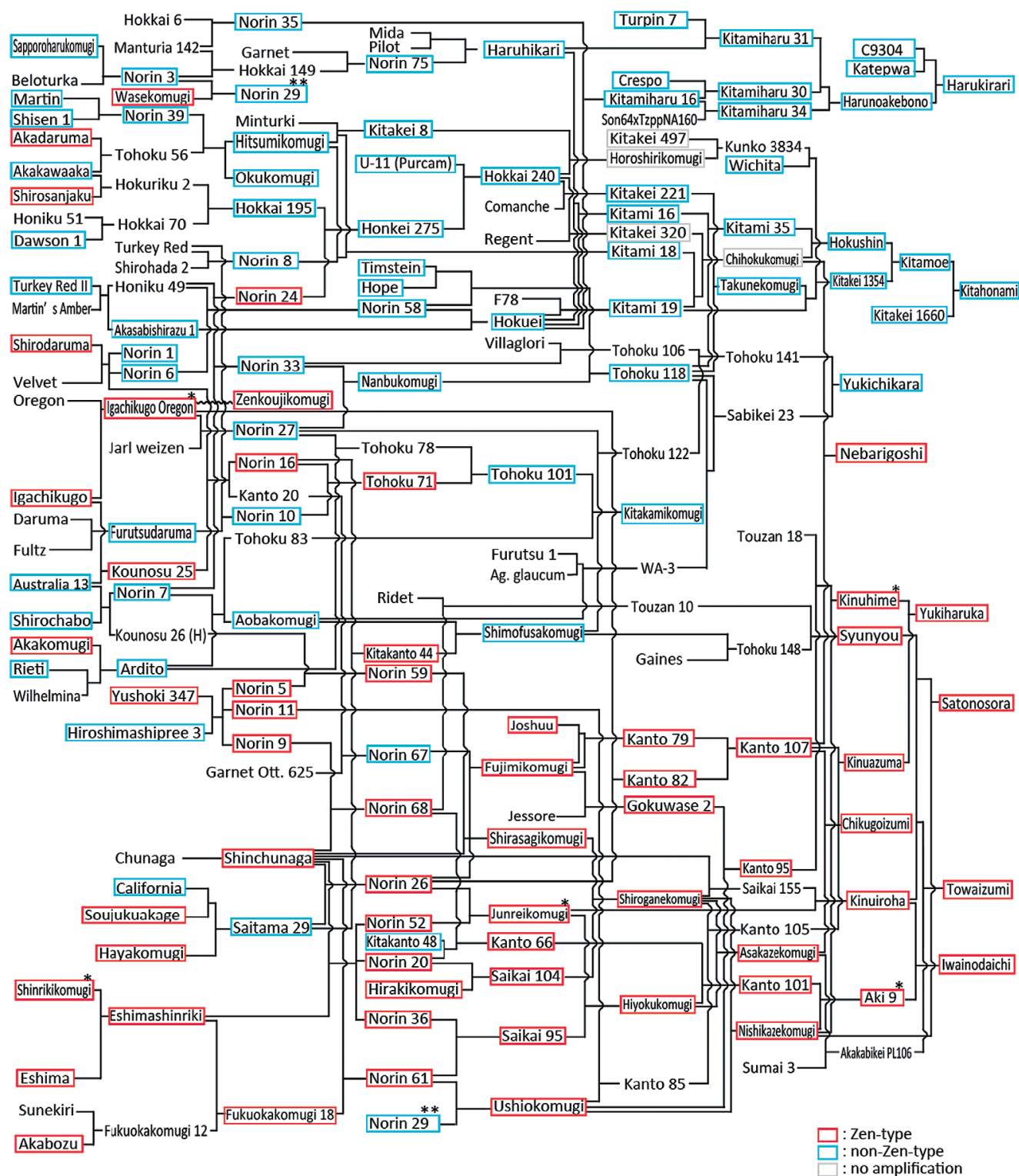
**Fig. 2.** Frequency distribution of Japanese wheat varieties for GI grown in 2004/2005, 2005/2006, and 2006/2007 growing seasons. Sample size (n) and the average GI values for the varieties carrying the Zen-type SNP (solid columns) and non-Zen-type SNP (open columns) are shown. A black arrowhead indicates the GI value for ‘Zenkoujikomugi’.



‘Zenkoujikomugi’. The GI values and the origin of these varieties are summarized in **Table 1**. All these varieties carry the Zen-type SNP and were developed and/or originated

in the southern part of Japan.

Frequency distribution of the GI values for the varieties carrying the Zen-type SNP and non-Zen-type SNP is shown



**Fig. 3.** *MFT-3A* genotype in the pedigree of varieties with strong grain dormancy. Red and blue frames indicate the varieties carrying the Zen-type SNP and non-Zen-type SNP, respectively. Gray frames indicate varieties with no amplification with the CAPS marker. A single asterisk indicates varieties with lower average GI values than ‘Zenkoujikomugi’. ‘Norin 29’ (double asterisk) is shown separately in the top and bottom sections of this figure. A wavy line indicates mutation breeding.

in **Fig. 2**. Four varieties with no amplification with the CAPS marker were excluded. The average GI values of the varieties carrying the Zen-type SNP and non-Zen-type SNP were 62.3 and 85.9, respectively, for 2004/2005 wheat-growing season. Similar genotype difference was observed in other growing seasons. The average GI values of the varieties carrying the Zen-type SNP were 66.3 (2005/2006) and 66.3 (2006/2007), and the average GI values of the varieties carrying the non-Zen-type SNP were 88.8 (2005/2006) and 91.3 (2006/2007). The correlation between *MFT-3A* genotype and grain dormancy was significant in three seasons ( $P < 0.001$ ). The GI values of the varieties carrying Zen-type SNP were widely spread. A number of QTLs related to the PHS tolerance and/or dormancy have been reported in wheat (reviewed in Gao *et al.* 2013, Kulwal *et al.* 2010). Other genes related to the regulation of PHS tolerance and/or dormancy are supposed to have an effect on grain dormancy in each variety.

### Genealogical pedigree of the varieties with strong grain dormancy in Japan

Among commercial varieties, ‘Iwainodaichi’ (Kyushu), ‘Junreikomugi’ (Kinki-Chugoku-Shikoku), ‘Kinuhime’ (Kanto-Tokai), ‘Nebarigoshi’ (Tohoku-Hokuriku), and ‘Kitamoe’ (Hokkaido) showed the lowest average GI value in each geographical group (**Supplemental Table 1**). *MFT-3A* genotypes in the pedigree of these varieties are summarized in **Fig. 3**. ‘Igachikugo Oregon’, which showed the lowest average GI value, is a parent of the variety ‘Zenkoujikomugi’. ‘Zenkoujikomugi’ was derived from ‘Igachikugo Oregon’ by mutation breeding using gamma rays (**Fig. 3**). ‘Aki 9’ showed strong grain dormancy and is a cross parent of ‘Iwainodaichi’ developed in the Kyushu region (**Fig. 3**).

The varieties carrying the Zen-type SNP were mainly developed in the Kyushu, Kinki-Chugoku-Shikoku, and Kanto-Tokai regions (**Fig. 1**, **Supplemental Table 1**). As shown in **Fig. 3**, the old varieties carrying the Zen-type SNP, such as ‘Yushoki 347’, ‘Eshimashinriki’, and ‘Shinchunaga’, were used as cross parents of the old Norin varieties in these regions. In particular, ‘Shinchunaga’ was used as a cross parent of 22 Norin varieties, of which 20 varieties (‘Norin 20’, ‘Norin 26’, ‘Norin 30’, ‘Norin 32’, ‘Norin 34’, ‘Norin 36’, ‘Norin 43’, ‘Norin 47’, ‘Norin 48’, ‘Norin 49’, ‘Norin 50’, ‘Norin 52’, ‘Norin 57’, ‘Norin 60’, ‘Norin 61’, ‘Norin 64’, ‘Norin 68’, ‘Norin 73’, ‘Shirasagikomugi’, and ‘Mikunikomugi’) were found to carry the Zen-type SNP. Thus, the varieties carrying the Zen-type SNP were distributed in the southern parts of Japan.

In the Tohoku-Hokuriku region, the trend in the *MFT-3A* genotype of commercial varieties has changed significantly. Only 8 of the 46 commercial varieties were found to carry the Zen-type SNP (**Fig. 1**). Among them, 5 of the 8 commercial varieties carrying the Zen-type SNP have been developed after 2000 (‘Nebarigoshi’, ‘Haruibuki’, ‘Mochihime’, ‘Ginganochikara’, and ‘Yukiharuka’). In recent years, a variety developed in the southern parts of Japan has been used

**Table 2.** Evaluation of grain dormancy in commercial varieties developed in the Hokkaido region

Name	<i>MFT-3A</i> Genotype	Germination Index	
		2006/2007	2007/2008
Horoshirikomugi	no amplification	73.5	86.9
Takunekomugi	non-Zen-type	82.9	57.6
Chihokukomugi	no amplification	92.5	82.9
Hokushin	non-Zen-type	85.7	69.8
Kitamoe	non-Zen-type	66.0	45.0
Kitahonami	non-Zen-type	11.4	11.9

as a cross parent in this region. For example, ‘Nebarigoshi’ is selected from double haploid lines of the cross of ‘Kanto 107’/‘Chihokukomugi’ (**Fig. 3**). ‘Kanto 107’ carrying the Zen-type SNP is a breeding line developed in the Kanto-Tokai region and shows a moderately strong dormancy (**Supplemental Table 1**). ‘Yukiharuka’ is developed from the cross between ‘Kinuhime’ and ‘Kinuazuma’, both of which carry the Zen-type SNP and were developed in the Kanto-Tokai region (**Fig. 3**). The newly developed varieties carrying the Zen-type SNP may, in part, be useful to improve the PHS tolerance of an upcoming variety in the Tohoku-Hokuriku region.

At the time when we started evaluating grain dormancy in Japanese wheat varieties, ‘Kitahonami’, which is now becoming a leading variety in Japan, had not been developed. To evaluate the grain dormancy of ‘Kitahonami’, the commercial varieties developed in the Hokkaido region were subjected to a germination assay for two wheat-growing seasons. As shown in **Table 2**, the GI value of ‘Kitahonami’ was the lowest in each season. In comparison with ‘Kitamoe’, the dormancy level was greatly improved in ‘Kitahonami’. None of the varieties in the Hokkaido region carried the Zen-type SNP, and the strong dormancy in ‘Kitahonami’ may be derived from other gene(s) that regulate grain dormancy. To examine the effect of the Zen-type SNP on grain dormancy in the Hokkaido region, we are preparing a new variety carrying the Zen-type SNP in a ‘Kitahonami’ background. Our future studies will clarify whether the Zen-type SNP in the promoter of *MFT-3A* improves the grain dormancy in varieties developed in the Hokkaido region.

### Acknowledgements

We acknowledge Dr. S. Taya for his helpful comments. We thank M. Nakamura, K. Yokoi, K. Suzuki, C. Amemiya, M. Someya, A. Yamase, and Y. Maki at NARO Institute of Crop Science for their technical assistance; the members of the field cultivation team at NARO Agricultural Research Center for cultivation of the experimental material; and Dr. H. Hanai for his helpful comments and ongoing support. This work was supported by Grants-in-Aid from NARO.

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