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Full Length Article

Evaluation of Functional Markers Related to Agricultural Traits for Selecting Efficient Marker Sets in Wheat

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Abstract

Agronomic traits of wheat like stem length, heading date and maturity are closely related to productivity of wheat. The assessment and database of molecular marker for the allelic variation of functional gene is very important to select useful alleles in wheat molecular breeding. Therefore, we analyzed 24 functional markers related to dwarf genes (*RhtB1* and *Rht D1*) associated with stem length, Photoperiod (*Ppd-1*) and vernalization gene (*Vrn1*) associated with heading and flowering time to improve the selection efficiency for 608 wheat genotypes originated from Asia, America and Europe. Through the association analysis of functional marker with agronomic traits, we confirmed genotypes of each genes and selected high efficiency 11 functional markers. *RhtB1a* (Rht1) and *RhtD1a* (Rht3), four *Ppd-1* (PPD1, 2, 3 and 5) and five *Vrn1* gene markers (Vrn1, 4, 7, 11 and 15) were highly correlated with each agronomic trait, plant height, heading date and maturity, respectively. Consequently, 11 functional markers identified, may be very useful as selection markers for wheat molecular breeding. © 2020 Friends Science Publishers.

Keywords: Molecular marker; Ppd; Rht; Vrn gene; MAS

Introduction

Wheat (*Triticum aestivum* L.) is one of the major food crops such as rice and corn (Howell *et al*., 1995). Common wheat has three different genomes A, B and D derived from tetraploid species *Triticum turgidum* L. and *Aegilops tauschii*, repectively (Matsuoka, 2011). More than 40% of the world's population use wheat as staple food, so wheat productivity is critical to human survival (Gupta *et al*., 2008). Abnormal climate change can affect agronomic traits like plant height or flowering and ripening period of wheat and lead to reduced productivity. The stem length and flowering date are closely linked wheat's productivity (Ul-Allah *et al*., 2018). Therefore, securing various wheat resource, characterizing them and analyzing related gene is very important in breeding program. Recently, researches on synthetic or hybrid wheat to conducted to collect and evaluate various wheat resources (Ahmad *et al*., 2019). In Korea, wheat breeding is carried out not only for wheat seed characteristics, but also for wheat varieties suitable for domestic agricultural systems and climatic conditions to speed up the maturation phase, leading to early harvest (Son *et al*., 2017). With the recent development of molecular breeding, genetic markers directly associated with wheat's agricultural characteristics have been developed and used to classify major germplasm resources containing Korean wheat varieties (Shin *et al*., 2014).

The semi-dwarf gene (Reduced height, *Rht*) is a very important gene that regulates the stem length of wheat and each allele has a different influence on the plant's length growth (Wilhelm *et al*., 2013; Casebow *et al*., 2016). In particular, the *Rht-B1b* and *Rht-D1b* genotypes show insensitivity to gibberellic acid (GA3), as they have the early termination codon formed by mutating the single nucleotide present in the DELLA domain. The DELLA protein is encoded by three genes, (*i.e*., the *Rht-A1*, *Rht-B1* and *Rht-D1*) that are present in chromosome 4 of the A, B, and D genomes (Wilhelm *et al*., 2013). Since an inactive protein with N-terminal deformity is generated, even when GA exists, it suppresses plant growth (Peng *et al*., 1999). Recently, genes causing a decreased height of wheat such as *Rht-B1c*, *Rht-B1d*, *Rht-B1e*, *Rht-D1c* and *Rht-D1d* have been reported (Wilhelm *et al*., 2013; Van De Velde *et al*., 2017). Since these genes have been reported to be involved in high temperature tolerance, they are very useful genes for developing crops that respond to climate change (Flintham *et al*., 1997).

The flowering of wheat can be promoted through a low-temperature acclimation process that regulates the temperature and day length and the main composition of

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low-temperature purification, *i.e*., the vernalization, is different for each wheat resource (Stelmakh, 1998; Fu *et al*., 2005). There are two main genes, *i.e*., *Vrn-1* and *Ppd-1*, which are involved in the vernalization and photoperiod, respectively. The dependence of flowering on day length can be explained by photoperiod sensitivity and photoperiod response in wheat is mainly determined by the homoeologous series of *Ppd-1* genes (Muterko *et al*., 2016).

VRN1 is suppressed as a flowering promoting factor until the low temperature condition is satisfied and *VRN2* is regulated by vernalizaion and a short photoperiod as a strong flowering inhibiting factor. The expression of *VRN3* is induced under the condition of a long photoperiod and it is known as a gene that accelerates reproductive growth (Distelfeld *et al*., 2009; Trevaskis, 2010). These genes are present on chromosome 5 as a homologous gene (*i.e*., homozygous) in each genome, and it has been reported that *VRN1* was very closely related to wheat flowering under various photoperiod conditions (Law *et al*., 1976; Stelmakh, 1987; Galiba *et al*., 1995).

The wheat including the photoperiod insensitive type allele, *Ppd-Ala*, can hasten the harvesting period (Guedira *et al*., 2016). The photoperiod insensitive type wheat with an early heading and flowering date has been bred for early harvest before the high temperature season arrives. When the vernalizaion is satisfied, the wheat growth stage changes to a reproductive stage by increasing the temperature. Therefore, it could shorten the cultivation period of wheat (Guedira *et al*., 2016).

Although, many molecular markers have been developed using gene or genome analysis, but there are inefficient or incorrect molecular markers exist. Therefore, in this research, in order to use genetic resources efficiently to develop wheat varieties suitable for the climate of Korea, functional markers related to plant height gene (*Rht1*), photoperiod gene (*Ppd-1*) and vernalization (*Vrn1*) were evaluated, because these genes are so closely linked to wheat productivity. In addition, a comparative analysis of these genotypes and agricultural traits such as heading date and maturity was carried out to characterize the suitable molecular marker as an indicator for breeding selection.

Materials and Methods

Plant Material Evaluation

The materials used in this study consisted of 608 accessions mostly originating from Korea, but also from some other countries like China, Japan, USA, Turkey and Mongolia. Each plot consisted of three 4.0 m rows spaced 25 cm apart in the experimental field of National Institute of Crop Science (NICS), Rural Development Administration (RDA) from 2015 to 2016. Sowing was carried out on October 25. Fertilizer was applied at 5: 7: 5kg/0.1ha (N: P: K) before sowing, and weeds, insects and diseases were stringently controlled. The agricultural traits like as stem length, heading and maturation date were investigated according to the Agricultural Science and Technology Survey Analysis Standard, RDA (RDA, 2012). The stem length measured the length of the part except the spike, and the heading date was decided when more than 40% of spike of the populations were appeared. The mature stage was made available for harvest.

Genomic DNA Isolation and PCR

The young leaves of wheat were immediately frozen with liquid nitrogen and then powdered using a plastic grinder. Genomic DNA was extracted from leaf powder (200 mg) using a plant genomic DNA prep kit (Solgent, Korea). The extracted genomic DNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). For the PCR, the Veriti® Dx 96-Well Thermal Cycler (Applied Biosystem, USA) was used. For the PCR reaction, 1 unit of Taq polymerase (Genotbio Prime DNA polymerase, Korea) was used for 100 ng genomic DNA, 1× PCR buffer, 0.5 uM primer, 200 uM, dNTP and the total reaction volume was 25 uL. The functional markers (FM) based on three genes, *Rht*, *Ppd* and *Vrn*, used in this study were selected from the Wheat Applied Genomics (http://maswheat.ucdavis.edu) (Table 1). The PCR condition was carried out after the denaturation reaction at 94°C for 5 min and then at the amplification step for 30 sec at 94°C, 30 sec at annealing temperature between 55°C and 65°C and 60 sec at 72°C. The PCR product was electrophoresed at 100V for 2 h with a 2.0% agarose gel containing EtBr (0.5 ug/uL) and then confirmed by a UV transilluminator.

Genotyping and Statistical Analyses of Trait Data

The amplified and non-amplified or non-targeted bands in the agarose gel were recorded as the binary codes “1” and “0”, respectively, which were used to analyze the correlation between the phenotypes and genotypes of the accessions or species. And “1” and “0” were transformed “a” and “b”, respectively, to consistently explain the polymorphism of functional marker in the result sections. “a” and “b” have a same meaning as “1” and “0”. Statistical analysis of the data was performed by R software (ver. 3.5.1) using analysis of variance (ANOVA), Duncan test and Pearson’s correlation coefficient. An overall mean dataset was generated for three agricultural traits like as stem length, heading and maturation date by averaging trait data over all replicates. ANOVA was carried out using quantitative variables for phenotype and genotype markers, and boxplot was indicated for the number of days according to each marker. Correlation analysis was used to investigate potential genetic relationships between the agricultural traits. Pearson's correlation coefficients were estimated between the stem length, heading date, maturation date, and genotypes of *rht*, *ppd* and *vrn* genes with procedure

R program using the 608 wheat resources from the overall mean data set. Pearson's correlation coefficients were also determined and statistical significance levels were $P<0.05$ unless otherwise specified.

Results

Relationship between Stem Length and *Rht* Gene

The characteristics and agricultural traits of major plants were investigated on 607 resources, excluding one line, the 17WCB007 (Shinkwang), which did not germinate among the resources of the wheat artificial mating group (608 accessions) possessed by the NICS. The seedling establishment of genetic resources, cold resistance, and growth was good, and the shape of the plant was classified into semi-open, semi-upright, and vertical types. There were six semi-open, 461 semi-upright, and 140 vertical type resources. The color of the leaf appeared in brown, light brown, green tea, red bean, and yellowish white. The leave color of wheat lines was mostly green (563 lines), and other lines were dark green (23 lines) and light green (21 lines). The color of the spikes was divided into brown, light brown, reddish brown, and yellowish white. Most of the resources were yellowish white with seven brown, nine light reddish brown, and 83 reddish brown. The stem length was 80 cm on average (minimum 46 cm, maximum 130 cm). the average length of the spike was 8.5 cm (minimum 4.8 cm, maximum 18.4 cm) and the average length of the awn was 5.8 cm (minimum 0.0 cm, maximum 12.7 cm). The average number of grains per spike was 48 (at least 24 grains, maximum 101), and the average number of spikes per m2 was 639/m2 (minimum 33, maximum 1,600).

The *Rht* gene associated with the wheat semi-dwarfing of 608 genetic sources was analyzed by using four primer sets, *i.e.*, Rht1, Rht2, Rht3 and Rht4 (Table 1). Four markers were included in the *Rht-B1* and *Rht-D1* genes: Rht1 in *Rht-B1a* (wild type), Rht2 in *Rht-B1b* (dwarf), Rht3 in *Rht-D1a* (wild type), and Rht4 in *Rht-D1b* (dwarf). Rht1, Rht2 and Rht3 were dominant molecular markers, while Rht4 was a recessive marker (Ellis *et al*., 2002). Out of the four molecular markers, Rht4 was not amplified in 608 resources.

The polymorphism analysis for each marker was performed using the PCR results of the four molecular markers. As a result of analyzing the stem length and polymorphism of the molecular markers, Rht1 showed an average length of a and b type, which was 81.4 and 77.5 cm, respectively. Rht2 showed 79.7 and 79.4 cm, respectively. Rht3 was found to be 75.5 and 80.1 cm, respectively, indicating that the stem length of the a-type was short. Rht1 and Rht3 showed opposite results. The stem length was longer in the genotype a of Rht1, and it was longer in the genotype b of Rht3. The difference was 4-5 cm. The stem length of wheat presenting Rht1 and Rht3 was longer than Rht2 and Rht4 (Table 2). However, in the correlation analysis, the four molecular markers such as Rht1, Rht2, Rht3, and Rht4 did not significantly correlate with the stem length of wheat (Table 4).

When four markers from Rht1 to Rht4 were sequentially represented, eight polymorphic combinations such as a/a/a/b were confirmed. With the exception of the a/b/b/b and b/a/b/b, the other variation ranges of combinations became very large (Fig. 1). The line (b/b/b/b) was not expressed in both bands of the four markers, which showed an average stem length of 77.3 cm and it included 48 resources. The combination of b/b/b/b did not have three predominant molecular markers such as *RhtB1a*, *RhtB1b*, and *RhtD1a*. Instead, it only had a recessive marker *RhtD1b* and showed an average stem length of 77.32 cm. When compared with resources that have these genotypes, the combination of a/b/b/b showed an average of 77.34 cm and the combination of b/b/a/b was 80.96 cm. All three expressed lines (a/a/a/b) of Rht1, Rht2, and Rht3 were 136, and the average stem length was 82.6 cm. The 489 (80.4%) of the resources (*RhtB1b/RhtD1b*) were not simultaneously expressed from Rht2 and Rht4 markers and 119 (19.6%) of the resources (*RhtB1a*/*RhtD1b*) were not expressed by Rht2. The combination with the shortest stem length was confirmed at 76.64 cm and 76.95 cm on average of a/a/b/b and b/a/a/b, respectively. All of these resources had both *RhtB1b* and *RhtD1b* at the same time. In contrast, the longest stem length combination was confirmed at a/b/a/b and a/a/a/b at 88.26 cm and 82.60 cm, respectively. These resources had *RhtB1a* and *RhtD1a* at the same time (Supplementary Table 1). However, the variation between each combination of the molecular markers and the stem length became very large (Fig. 1).

Relationship between Heading Date and *Ppd-1* Gene

The heading date was on average May 1 (at least April 15, maximum April 27). Analysis of the photoperiod-1 (*Ppd-1*) genes involved in the wheat maturation period was carried out using five functional markers called the PPD1, PPD2, PPD3, PPD4, and PPD5. These markers were related to *Ppd-B1* and *Ppd-D1* (Table 1). Four polymorphic bands were confirmed in PPD4, and one polymorphic band was confirmed in the other four. Therefore, PPD4 did not contain data in the combinatorial analysis of polymorphism. As a result of analyzing the relationship between the polymorphism of each molecular marker and heading date, the average heading dates for PPD1 type a and type b were May 3 and April 29, respectively. Other results were represented to Table 3.

By using the PCR results of the four molecular markers PPD1, PPD2, PPD3 and PPD5, when a and b were written in turn, the combination of 16 polymorphisms such as a/a/a/b was confirmed (Supplementary 2). The variation of the relationship between the molecular marker polymorphism and heading date of each PPD became very broad (Fig. 2).

Table 1: The information of functional markers to analyze genetic polymorphisms

No.	Gene	Primer name	Forward primer(5'→3')	Reverse primer(5'→3')
1	Rht-B1	Rht1	GGTAGGGAGGCGAGAGGCGAG	CATCCCCATGGCCATCTCGAGCTG
2	Rht-B1	Rht2	CCAGATACACAACTGCTGGC	TGATCTTGAGGTTCTCGTCG
3	Rht-D1	Rht3	GGCAAGCAAAAGCTTCGCG	GGCCATCTCGAGCTGCAC
4	Rht-D1	Rht4	CGCGCAATTATTGGCCAGAGATAG	CCCCATGGCCATCTCGAGCTGCTA
5	Ppd1	PPD1	AGGCTCTTTGGCTATGACG	ATTTCCAACGTTACAAGTGGG
6	Ppd2	PPD2	AGGCTCTTTGGCTATGACGT	ATTCCAACGTTACAAGTGGA
7	Ppd3	PPD3	CCCAATATCTACTCCTCCGC	TCTGAATGATGATACACCATG
8	Ppd-D1	PPD4	ACGCCTCCCACTACACTG	CACTGGTGGTAGCTGAGATT
9	Ppd-D1	PPD5	ACGCCTCCCACTACACTG	GTTGGTTCAAACAGAGAGC
10	VRN-A1	vm1	GAAAGGAAAAATTCTGCTCG	GCAGGAAATCGAAATCGAAG
11	VRN-A1	vm2	GTTCTCCACCGAGTCATGGT	AAGTAAGACAACACGAATGTGAGA
12	VRN-B1	vm3	CAAGTGGAACGGTTAGGACA	CTCATGCCAAAAATTGAAGATGA
13	VRN-B1	vm4	CAAGTGGAACGGTTAGGACA	CAAATGAAAAGGAATGAGAGCA
14	VRN-B1	vm5	CCCCTGCTACCAGTGCCTACTA	CCCGCTTGTTGGCTGGTGAG
15	VRN-B1	vm6	CCCCTGCTACCAGTGCCTACTA	GCCCCATCTCCGCTGGAGAACG
16	VRN-D1	vm7	GTTGTCTGCCTCATCAAATCC	GGTCACTGGTGGTCTGTGC
17	VRN-D1	vm8	GTTGTCTGCCTCATCAAATCC	AAATGAAAAGGAACGAGAGCG
18	VRN-H1	vm9	GCTCCAGCTGATGAAACTCC	CTTCATGGTTTTGCAAGCTCC
19	VRN-H1	vm10	TTCATCATGGATCGCCAGTA	AAAGCTCCTGCCAACTACGA
20	VRN-B3	vm11	CATAATGCCAAGCCGGTGAGTAC	ATGTCTGCCAATTAGCTAGC
21	VRN-B3	vm12	ATGCTTTCGCTTGCCATCC	CTATCCCTACCGGCCATTAG
22	VRN-D1	vm13	GTTGTCTGCCTCATCAAATCC	CTCATGCCAAAAATTGAAGATGA
23	VRN-D1	vm14	GCACTCCTAACCCACTAACC	TCATCCATCATCAAGGCAAA
24	VRN-A1	vm15	AGCCTCCACGGTTTGAAAGTAA	AAGTAAGACAACACGAATGTGAGA

Table 2: Differences in stem length depending on the polymorphisms of Rht-1 functional markers

Primer name	Polymorphism	Average of stem length (㎝)	SD	Number of lines	Alleles
Rht1	a	81.4	16.73	319	*Rht-B1a*
	b	77.7	17.11	288	
Rht2	a	79.7	16.9	489	*Rht-B1b*
	b	79.4	17.48	118	
Rht3	a	75.51	15.34	64	*Rht-D1a*
	b	80.15	17.13	543	
Rht4	a	-	-	0	*Rht-D1b*
	b	80.1	0	607	

SD; standard deviation
a indicate amplified and b indicate non-amplified band in PCR reaction

Table 3: Differences in the heading date depending on the polymorphisms of Ppd-1functional markers

Primer name	Polymorphism	Average of heading date (mm.dd.)	SD	Number of lines	Alleles
PPD1	a	05. 03.	7.17	255	*Ppd-B1a*
	b	04. 29.	7.66	352	
PPD2	a	05. 05.	8.95	79	*Ppd-B1a*
	b	04. 30.	7.29	528	
PPD3	a	05. 01.	7.72	359	*Ppd-D1a*
	b	04. 30.	7.65	248	
PPD4	a	04. 27.	7.73	222	*Ppd-D1a*
	b	05. 07.	7.65	385	
PPD5	a	05. 05.	7.73	222	*Ppd-D1a*
	b	04. 28.	7.65	385	

SD; standard deviation
a indicate amplified and b indicate non-amplified band in PCR reaction

The combination of the most polymorphisms among the analyzed genetic resources was the combination expressed as b/b/a/b, and 120 resources were included. With the combination of 16, nine combinations such as a/a/b/b (April 30) were confirmed for the combination earlier than the average heading date (May 1). The resources expressed in all four bands (a/a/a/a) were 23 and its heading date was confirmed late on May 9. On the contrary, all of the unexpressed resources (b/b/b/b) were 115. The heading date of 44 lines that had the b/a/b/b combination was the fastest on April 23 (Supplementary 2). PPD2 and PPD5 showed a positive correlation, and PPD5 showed a strong negative relationship. Among the four markers, PPD1, PPD2, and PPD5 were highly related to wheat heading. PPD1 and PPD2 also showed a weak negative correlation. In particular, the correlation between PPD1/PPD5 and PPD2/PPD3 was high. In combining the two markers, a/a was the earliest heading date, and b/b was the fastest (Table 4).

In the case of PPD3, it was considered that the difference between the genes of type a and b hardly affected

Table 4: Correlation coefficients between the agricultural traits and molecular markers of wheat resources

	Rht1	Rht2	Rht3	PPD1	PPD2	PPD3	PPD5	vrn1	vrn2	vrn3	vrn4	vrn5	vrn6	vrn7	vrn8	vrn9	vrn10	vrn11	vrn15
Rht2	0.117																		
Rht3	0.138	0.090																	
PPD1	0.394	0.140	0.339																
PPD2	0.083	0.017	0.204	0.008															
PPD3	0.002	0.125	0.130	0.062	0.172														
PPD5	0.187	0.010	0.310	0.158	0.316	0.081													
vrn1	0.114	0.098	0.158	0.150	0.132	0.140	0.191												
vrn2	0.202	0.258	0.172	0.281	0.112	0.276	0.141	0.211											
vrn3	0.175	0.302	0.186	0.277	0.105	0.271	0.112	0.225	0.453										
vrn4	0.091	0.240	0.010	0.065	0.127	0.257	0.105	0.117	0.324	0.310									
vrn5	-0.033	0.143	0.009	-0.060	0.039	0.101	-0.038	0.126	0.090	0.187	0.271								
vrn6	0.129	0.259	0.172	0.205	0.148	0.415	0.182	0.280	0.455*	0.460*	0.322	0.156							
vrn7	0.048	0.106	0.095	0.003	0.221	0.136	0.236	0.216	0.327	0.237	0.163	0.136	0.232						
vrn8	-0.122	0.152	-0.202	-0.494*[a]	-0.010	0.229	-0.088	-0.064	0.154	0.129	0.146	0.104	0.101	0.344					
vrn9	0.016	0.032	0.114	0.181	-0.025	0.060	-0.043	-0.123	0.019	0.082	-0.052	0.036	0.051	-0.096	-0.135				
vrn10	-0.007	0.034	0.017	-0.119	0.195	0.118	0.091	0.201	0.215	0.166	0.119	0.158	0.156	0.378	0.227	-0.148			
vrn11	0.067	0.002	0.143	0.260	-0.023	-0.064	0.066	-0.061	0.073	0.096	-0.546*	-0.272	0.100	-0.023	-0.107	0.165	-0.065		
vrn15	0.027	0.080	-0.037	0.076	-0.029	0.121	-0.117	-0.364	0.159	0.122	0.106	0.063	0.063	-0.038	-0.014	0.063	0.074	-0.016	
Maturity	-0.100	0.055	-0.178	-0.226	-0.255	-0.035	-0.479*	-0.101	0.020	-0.039	-0.005	0.103	-0.115	-0.066	0.214	-0.045	0.041	-0.114	0.222
Stem length	-0.107	-0.006	-0.168	-0.199	-0.010	0.017	-0.378	-0.032	0.038	0.027	0.053	0.215	-0.080	0.035	0.252	0.026	0.134	-0.174	0.178
Heading	-0.105	0.051	-0.184	-0.248	-0.209	-0.033	-0.480*	-0.058	0.021	-0.045	0.025	0.117	-0.116	-0.068	0.244	-0.057	0.052	-0.144	0.196

[a] indicates significance at *P<0.05

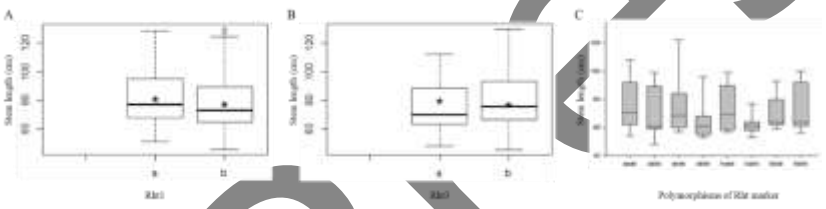

Fig. 1: Box plot between heading date and genotypes of each Rht functional marker or each combination. The genotype a means the amplified polymorphism and b is not the amplified polymorphism in each marker. A; Rht1, B; Rht3, C; combination of four Rht, Rht1, Rht2, Rht3 and Rht4 markers. The star (*) indicates the average location

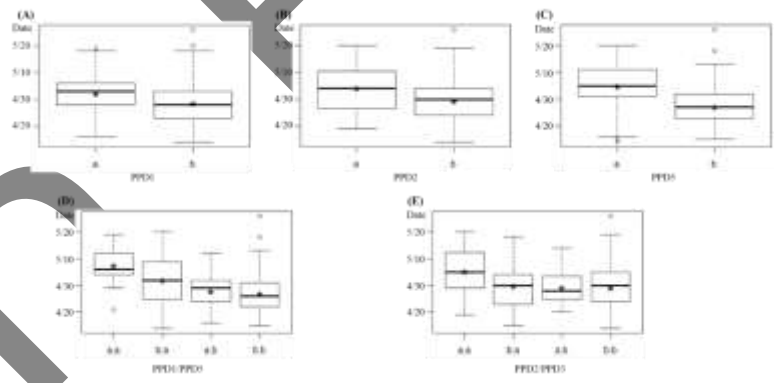

Fig. 2: Box plot between the heading date and genotypes of each PPD functional marker or each combination. The genotype a means amplified polymorphism and b is not amplified polymorphism in each marker. A) PPD1; B) PPD2; C) PPD5; D) combination of PPD1 and PPD5 markers; E) combination of PPD2 and PPD3. The star (*) indicates the average location

the heading of wheat as a day. Even if PPD2 (*PpdB1*) was expressed in type a when PPD5 expressed type b, it did not have a big influence on the heading date because it was shown to be the fastest one. It was found that when PPD1 was present in the allele of *PpdB1a*, the heading date became slow. In the cases of PPD4 and PPD5, they represent a strong positive correlation and negative correlation, respectively, so it was expected that their effect will be larger than that of PPD1. Also, PPD4 and PPD5 showed a strong correlation with the stem length, heading date and maturity. Such results indicate that the stem length, heading date, and maturity of wheat were very closely related. Among them, the heading date and the maturity showed a very high correlation (0.884).

Relationship between Maturity and *Vrn* Gene

The maturity was on average June 6 (at least May 27,

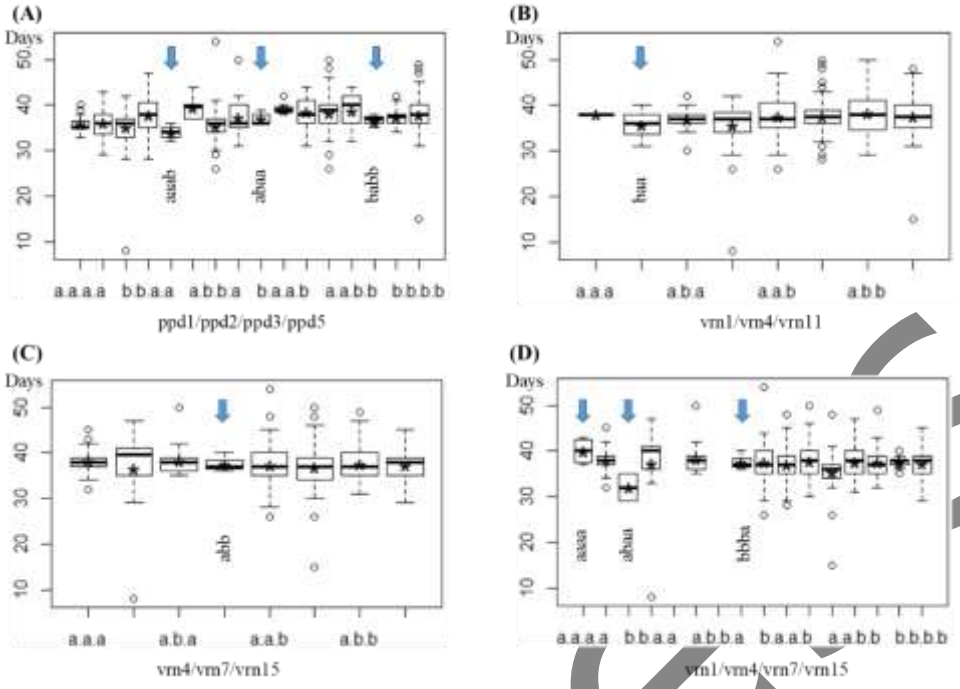

Fig. 3: Box plot between the days from the heading date to maturity (dH-M) and the genotypes of PPD and the vrn functional marker combination. The genotype a means amplified polymorphism and b is not the amplified polymorphism in each marker. The arrows indicate the selected marker sets. The functional marker combination of PPD1/PPD2/PPD3/PPD5(A), vrn1, vrn4, vrn11(B), vrn4, vrn7, vrn15(C), vrn1, vrn4, vrn7, and vrn15(D). The star (*) indicates the average location

maximum June 23). The analysis of polymorphism used a total of 15 primers, including vrn1 to vrn15. Three of the 15 markers (vrn12, vrn13, and vrn14) were not amplified in all 608 resources. The combination of 201 polymorphisms from the genotyping of 12 markers were confirmed. Among them, 42 wheat lines included the combination of a/a/a/a/a/a/a/a/b/a/b/b, and its average maturity period was confirmed on June 12. Seven combinations such as a/a/a/a/a/a/a/b/a/b/b appeared faster than the average maturity period (June 7), and the two combinations of a/a/a/a/a/a/b/a/b/a and b/a/a/a/a/a/b/b/b/b/a were confirmed to be the most rapid on June 3 and June 4, respectively (Supplementary 3). In addition, it was confirmed through correlation analysis that vrn8 and vrn15 showed a weak positive correlation with wheat maturation, and the ten molecular markers, excluding the two markers, were not related to the maturation of wheat (Table 4).

The correlation analysis between the number of days from the heading to the maturity (dH-M) of the wheat and the marker was performed. The correlation between vrn8 and vrn15 was highly correlated between dH-M. However, the combination of vrn1 and vrn8 in the analysis according to marker combinations showed a high of dH-M. The a/a genotype of vrn8/vrn15 marker set was the shortest with 35.73 days, and the a/b genotype was the longest with 38.81 days, respectively. The combination of vrn10 and vrn11 was the shortest with an a/a type of 35.25 days than the other combination of 36-37 days. The b/a/a and b/a/a genotypes of the combination of vrn1, vrn4 and vrn11 showed shortly as 35.75 days and 35.76 days, respectively. The a/b/a/a and a/a/a/a genotypes of the combination of vrn1, vrn4, vrn7, and vrn15 were found to be the shortest and the longest at 32 days and 40 days, respectively (Fig. 3).

Discussion

Rht-1 dwarfing alleles are related to stem length as well as decreased seed size and protein content of wheat (Casebow *et al*., 2016). *Rht-B1b* and *Rht-D1b* alleles of *Rht-1* gene reduce plant height by 14 - 17% and decreased lodging and yield productivity (Rasheed *et al*., 2016). It has been reported that the wheat containing *Rht-1a* have longer stems, especially wheat varieties containing *Rht-B1a*/*Rht-D1a* (Peng *et al*., 1999, Ellis *et al*., 2002). In addition to *RhtB1* and *RhtD1*, the semi-dwarfing genes were also noticed in *Rht-B1c*, *Rht-B1d*, *Rht-Ble*, *Rht-D1c* and *Rht-D1d* (Wilhelm *et al*., 2013; Van De Velde *et al*., 2017). It was expected that even wheat with *RhtD1b* (Rht4, type b) and *RhtB1a* (Rht1, type a) had almost no effect on the stem length, but *RhtD1a* (Rht3, type a) had its wheat stem length increased. In addition, the wheat that had *RhtB1b* (Rht2, type a) and *RhtD1b* at the same time needed to have *RhtB1a* and *RhtD1a* (a/a/a/b) at the same time so that the stem length becomes longer, and when it had either of the genes, it was found that there was almost no change in the stem length. Such results confirmed the same results as presented

by Peng *et al*. (1999) and Ellis *et al*. (2002). *Rht-B1b* allele presence in 45% wheat accessions and most wheat cultivars are containing wild type *Rht-D1a* (Wurschum *et al*., 2017). So, *RhtD1b* was shown that it was like as a recessive gene, because *RhtD1b* allele (Rht4) did not detected in all of the collected resources in this study. In the case of *RhtB1b*, when there no *RhtB1a* and *RhtD1a* were present, it is considered that *RhtB1b* affected the stem length as its variation depends on the presence or absence of *RhtB1b*. These results suggest that *RhtB1b* has a greater influence on the stem length than the *RhtD1a* allele. It is believed that *RhtD1a* is more influential than *RhtD1b*. Therefore, in order to select resources for the short stem length based on the *RhtB1* and *RhtD1* genes as molecular markers in wheat breeding, it is best to not have the *RhtB1a* and *RhtD1a* alleles together or resources with the *RhtB1b* allele must be selected. In particular, it has been reported that *Rht-B1b* can increase the wheat production by about 24% and the *Rht-D1b* gene by 16% (Filntham *et al*., 1997; Singh *et al*., 2001). In addition to this, semi-dwarfing genes were associated with abiotic stress such as a high temperature drought, but there was also a report that the effect of the *Rht-B1b* and *Rht-D1b* genes was particularly low in the spring wheat (Shin *et al*., 2014). In the future, it is conceivable that more analyses such as the mutation and function of these genes are required, and research related to the characteristics of the main wheat such as productivity and resistance to abiotic stresses as well as the stem length of wheat is necessary.

Three genes controlling vernalizaton (*VRN1*), photoperiod response (*Ppd-1*) and early flowering *(Dlf3)* are an important that regulates the response in the field at the flowering stage, and the expression of *VRN1* increases under the condition of a long photoperiod (Kamran *et al*., 2014). *Ppd-B1* was associated with 1000 grain weight and *Vrn-A1* was associated with grain yield and 1000 grain weight (Boden *et al*., 2015; Ogbonnaya *et al*., 2017). *Ppd-1* gene identified various spices for alternated alleles. *Ppd-A1a*.1 and *Ppd-A1a*.4 alleles previously identified in *T. aestivum* and *Triticum compactum*, respectively (Nishida *et al*., 2013; Muterko *et al*., 2015), while such PI alleles as *Ppd-A1a.2* and *Ppd-A1a.3* were found in *T. durum* (Wilhelm *et al*., 2009). However, the expression pattern of this gene during the day was known to have no difference under short or long photoperiod conditions (Murai *et al*., 2003). Shimada *et al*. (2009) proposed that *VRN1* was regulated by a photoperiod that is expressed most abundantly when light is initiated in both conditions. The analysis of the association between *Ppd-1* and *VRN1* revealed that *VRN1* expression did not change on the basis of the photoperiod sensitive (*Ppd-1b*) and insensitive (*Ppd-1a*) alleles, but it was regulated by the process of the photoperiod that regulated the *Ppd-1* gene (Kitagawa *et al*., 2012). In addition, it has been reported that *VRN1* was directly or indirectly affected by the flowering inhibiting factor *VRN2* (Distelfeld *et al*., 2009; Shimada *et al*., 2009; Trevaskis, 2010). Also, in the case of barley, the alleles of *Ppd-H1* were found to have no effect on the expression of *VRN-H1*/*HvVRN1* (Campoli *et al*., 2012). Using these results, Campoli *et al*. (2012) suggested that the *VRN1* of wheat was not regulated by *Ppd-1*, but was regulated individually by other paths (Kitagawa *et al*., 2012). For *Ppd1* and *VRN1* correlation analyses between the *Ppd1* and heading date, *VRN1* and maturity were performed, indicating that the two genes are associated with the heading date and maturity. Based on the genotypes of five alleles such as *PpdB1* and *PpdD1*, it was found that the average heading date increased or decreased from 1 day to 10 days.

The heading date of the spike was affected by the photoperiod and temperature. Although wheat responds to the photoperiod, the heading of spike is delayed when the accumulated temperature is not met. Therefore, the heading of spike control by the *Ppd1* single gene could not be accurately accounted for. However, as in sensitive genes, *PpdB1b* and *PppD1b*, type b (non-amplified) is considered to be type a (amplified) for the insensitive genes, such as *PpdB1a* and *PppD1a*, which rapidly control the heading of the spike. Particularly in PPD4, a band of 288 bp was confirmed as the *PpdD1a* allele among the four polymorphic bands (Beales *et al*., 2007). *PpdD1a* and *PpdD1b* were considered to be major factors in wheat heading, and PPD4 and PPD5 strongly influenced the heading of wheat more than the other functional markers. Therefore, it is considered that genotypes of PPD1, PPD4, and PPD5 can be used as major factors of *PpdB1* and *PpdD1* when the line was selected based on the heading date in wheat breeding.

Genotypes and the relationship dH-M of the *Ppd-1* and *VRN1* functional marker were analyzed. In the ppd1 and ppd5 combinations, the a/a type showed the slowest heading, but dH-M showed the fastest with 35 days. This means that although the heading was late, it matured at a high rate and, as a result, reached the maturity of the average wheat. The a/a/b/a was the fastest with 34.0 days when analyzed with all of the genotypes such as ppd1/2/3/5. In addition, a/b/a/a, a/b/b/a, a/a/a/a was 35.0 days, a/a/a/b was 37.0 days and b/a/b/b was 36.6 days, respectively.

It is known that the *Vrn* gene affects not only the flowering but also the plant height and grain yield of wheat (Stelmakh, 1998; Ogbonnaya *et al*., 2017). In this study, the correlation between the wheat maturity and *Vrn* gene was analyzed, and it was found that vrn15 of 15 markers related the most to the maturity than the others. When vrn15 was present as type a, it was found that the maturity was earlier than type b. As for the next *VrnD1* allele, it was found that the type a of vrn8 had an earlier maturity. Other molecular markers showed no significant difference in the maturity due to their genotype. For example, vrn4 was associated with the rapid maturation of genotype b. It was considered effective to use the five markers, *i.e.*, vrn1, vrn4, vrn7, vrn11, and vrn15, as a set for the comprehensive selection of resources related to rapid heading and maturation.

Conclusion

Some functional markers that had a high correlation with each agricultural characteristic were selected, and the combination was constructed. For example, using the polymorphism of the three Rht functional markers, rht1, rht2, and rht3 was able to analyze the stem length of wheat, and using the nine functional markers like PPD1, PPD2, PPD3, PPD4, vrn1, vrn4, vrn7, vrn11, and vrn15 could be used to analyze the heading date and maturity. This process is also expected to be very efficient for wheat breeding. In particular, the characteristics of the days from the heading date to maturity were considered to be effective by using the two polymorphism sets of vrn1/vrn4/vrn11 and vrn1/vrn4/vrn7/vrn15, respectively.

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