Wheat Inf. Serv. 103, 2007. www.shigen.nig.ac.jp/ewis

Wheat Information Service

Electronic Newsletter for Wheat Researchers

No. 103

CONTENTS

Wheat Inf. Serv. 103: 1-4, 2007. www.shigen.nig.ac.jp/ewis

Research Information

A new co-dominant PCR-based marker to identify the high-molecular-weight glutenin subunit combination "5+10" of common wheat

Goro Ishikawa, Toshiki Nakamura*
Tohoku National Agriculture Research Center, Morioka, Iwate, 020-0198, Japan
*Corresponding author: Toshiki Nakamura (E-mail: tnaka@affrc.go.jp)

Abstract
Glutenin, which is one of the major storage proteins of wheat, is composed of high and low molecular weight subunits. The high-molecular-weight subunits encoded by the *Glu-A1*, *-B1* and *-D1* genes are closely related to bread-making quality. Each locus has several alleles, among which the *Glu-D1d* allele, responsible for producing the subunit combination defined as "5+10", has the greatest positive effect on bread-making properties. We developed a new PCR-based co-dominant marker to identify this allele. This marker can also be used for multiplex PCR along with previously developed markers for the selection of partial waxy wheat lines.

The bread-making quality of wheat flour depends on the quantity and quality of storage proteins. In particular, high-molecular-weight glutenin (HMWG) has a large effect on bread-making quality, and several types of HMWG subunits have been identified (Shewry 2003). The "5+10" subunit combination is known to have a positive effect on dough properties and most cultivars possessing this subunit profile show good bread-making quality (Shewry 2003).

Since most of the cultivars produced in Japan's wheat breeding programs were developed for "Udon" noodles, the strong dough properties required for bread-making were not a major focus. Only two cultivars with the "5+10" subunit combination were identified when the HMWG subunits of Japanese cultivars were analyzed twenty years ago. However, there is a high demand for new cultivars with good bread-making quality due to increasing interest in food safety and domestic food products. Therefore, in the last decade, breeders have made efforts to introduce the "5+10" subunit combination into Japanese wheat lines using the SDS-PAGE screening method. During the same time period, DNA marker assisted selection (MAS) has been introduced into several public breeding programs, including rice, vegetable, orchard plant, and wheat programs. In particular, MAS for partial waxy lines with low amylose has been widely using in breeding programs for noodle-making cultivars. This success has induced demand for the development of a DNA marker for MAS of the "5+10" subunit combination.

The HMWG subunits are encoded at three homoeologous complex *Glu-1* loci, *Glu-A1*, *-B1* and *-D1*. Each locus has two tightly linked structural genes encoding a larger x-type subunit and a smaller y-type subunit (Payne et al. 1981). Various alleles occur at each locus, and the "5+10" subunit combination is derived from the *Glu-D1d* allele (Payne and Lawrence 1983). Although PCR-based markers for a number of subunits have been developed (e.g. Ahmad 2000; de Bustos et al. 2001; Ma et al. 2003; Moczulski and Salmanowicz 2003), most of these, including the markers for the "5+10" subunit combination, are dominant markers that amplify target products only when the targeted allele exists. When we tested the markers designed for the "5+10" subunit combination, we could not get clear amplified target fragments of the reported sizes. Unfortunately, a non-specific amplification product of the same size as the expected fragment from the "5+10" allele was identified in lines which do not carry this allele. In addition, reproducibility was poor, although we carefully conducted PCR using the same procedure and solutions as outlined in the original reports. All markers were dominant, and as such, were not suitable for continuous backcross procedures because they could not distinguish heterozygous from wild type F_2 plants. To solve these problems, we attempted suitable

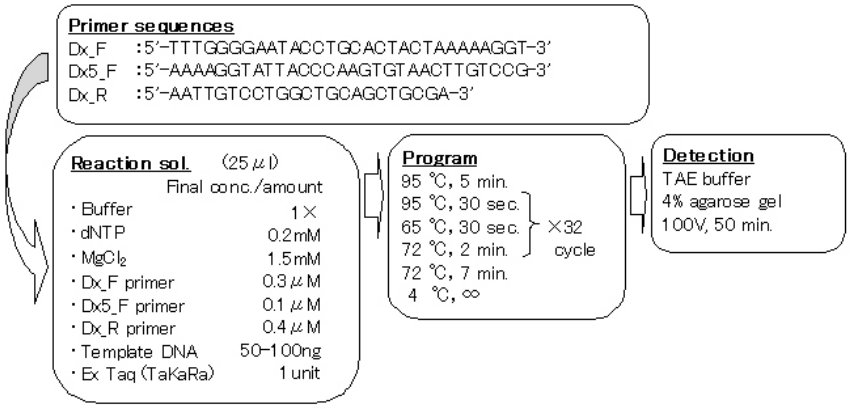

Figure 1. Primer sequences and PCR conditions. Three primers amplify two products of 343 and 320 bp when the *Glu-D1d* allele is present, while a 361 single product is amplified when this allele is absent.

for MAS of breeding lines carrying the "5+10" subunit combination.

Our first step was to align the sequences of all *Glu-D1* alleles deposited in GenBank databases. In contrast to other *Glu-D1* alleles, sequences derived from the *Glu-D1d* allele carried a single nucleotide substitution at position 353 and an 18-bp deletion at position 429 of the gene encoding the x-type subunit. Three primers were designed that amplified two products of 343 bp and 320 bp with genomic DNA from cultivars carrying *Glu-D1d*, while only a single product of 361 bp was amplified from cultivars carrying other alleles. The primer information and PCR conditions are shown in Fig. 1. Verification of the PCR marker was carried out with four cultivars, 'Haruyutaka', 'Takunekomugi', 'Harunoakebono' and 'Norin 61'. The HMWG subunit profile of these cultivars was determined by the SDS-PAGE method (Fig. 2), and only 'Harunoakebono' carried the *Glu-D1d* allele. As expected, two products of the predicted sizes were amplified from 'Harunoakebono' while only the 361-bp fragment was produced from the other cultivars (Fig. 3). Therefore, we concluded that the marker we developed could distinguish *Glu-D1d* from the other major *Glu-D1* alleles such as *Glu-D1a*, *Glu-D1c* and *Glu-D1f*. In addition, using DNA from the F_2 plants of a cross between 'Mori-kei C-130a' (with "2+12" subunit combination) and 'Tohoku 205' (with "5+10" subunit combination), we identified several plants which showed all three amplification products, indicating that these plants were heterozygous (Fig. 3, lane 8 and 9); these results were confirmed by SDS-PAGE analysis (data not shown). The stability, specificity and co-dominancy of the marker has been demonstrated through its use in several breeding programs in Japan.

Low amylose content in wheat flour appears to show a positive effect on bread-making quality by improving the texture and shelf life of bread (Lee et al. 2001; Morita et al. 2002). Amylose content is controlled by three homoeologous waxy (*Wx*) genes, *Wx-A1*, *-B1* and *-D1* (Nakamura et al. 1993; Chao et al. 1995) and the *Wx-B1* null allele significantly reduces amylose content (Miura and Sugawara 1996; Yamamori and Quynh 2000). Three cultivars recently

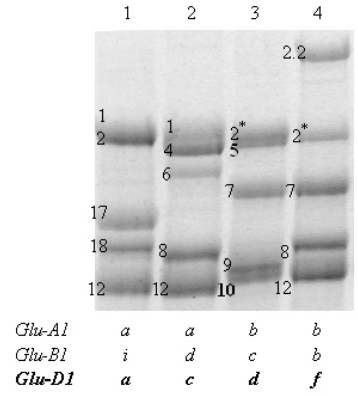

Figure 2. SDS-PAGE analysis of high-molecular-weight glutenin subunits. The *Glu-A1, B1* and *D1* alleles present in the four cultivars used in this work were determined by analysis of HMWG subunit patterns. Lane 1: 'Haruyutaka', 2: 'Takunekomugi', 3: 'Harunoakebono', 4: 'Norin 61'.

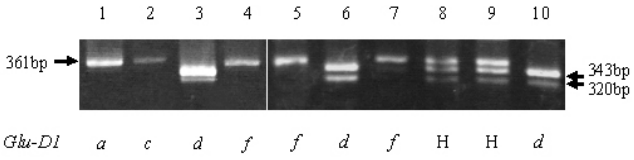

Figure 3. DNA marker identification of the *Glu-D1d* allele. Lane 1: 'Haruyutaka', 2: 'Takunekomugi', 3: 'Harunoakebono', 4: 'Norin 61', 5: 'Mori-kei C-130a' (with 2+12 subunit combination), 6: 'Tohoku 205' (with 5+10 subunit combination), 7-10: F_2 plants from Mori-kei C-130a/Tohoku 205. H: heterogeneous plant.

released in Japan, 'Haruhinode', 'Kitanokaori' and 'Haruyokoi', have the low amylose content induced by the null *Wx-B1* allele and also carry the "5+10" subunit combination. The acceptance of these cultivars by the flour and bread-making industry has strongly stimulated breeders to develop new cultivars with these properties.

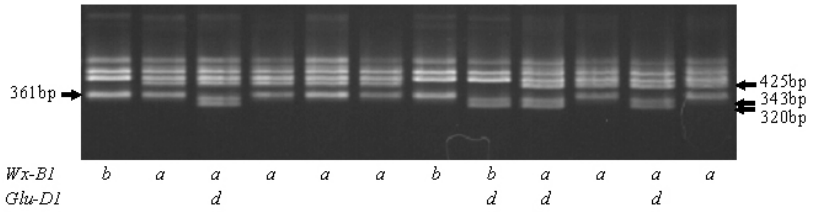

Figure 4. Multiplex PCR using primer sets capable of selecting for both the *Glu-D1d* and the null *Wx-B1* alleles. The 425 bp product is amplified from the wild type *Wx-B1a* allele but is not produced from the null *Wx-B1b* allele (Nakamura et al. 2002). The plant in lane 8 carries the *Glu-D1d* and null *Wx-B1* alleles.

PCR-based marker sets to identify null alleles of homoeologous *Wx* genes (Nakamura et al. 2002) have been adopted in Japanese breeding programs. The *Glu-D1d* marker was developed to work under the same conditions as the markers for the detection of *Wx* null alleles. This enabled us to conduct a multiplex PCR using the PCR conditions shown in Fig. 1. The multiplex PCR required only the addition of two primers for the *Wx-B1* null allele, each at a final concentration at 0.2 µM. With this reaction, all of the expected amplification products could be obtained and plants with both the *Glu-D1d* and *Wx-B1* null alleles could be identified (Fig. 4). Marker-assisted selection with DNA markers, as shown in this trial, allows us to select for several traits at once using a single DNA sample from each plant. There is no doubt that additional DNA markers suitable for breeding programs will be developed in the near future, providing an even greater advantage to breeders.

References

Ahmad M. 2000. Molecular marker-assisted selection of HMW glutenin alleles related to wheat bread quality by PCR-generated DNA markers. Theor Appl Genet 101: 892-896.

de Bustos A, Rubio P, Soler C, Garcia P, Jouve N. 2001. Marker assisted selection to improve HMW-glutenins in wheat. Euphytica 119: 69-73.

Chao S, Sharp PJ, Worland AJ, Koebner RMD, Gale MD. 1989. RFLP-based genetic maps of wheat homoeologous group 7 chromosomes. Theor Appl Genet 78: 495-504.

Lee M-R, Swanson BG, Baik B-K. 2001. Influence of amylase content on properties of wheat starch and breadmaking quality of starch and gluten blends. Cereal Chem 78: 701-706.

Ma W, Zhang W, Gale KR. 2003. Multiplex-PCR typing of high molecular weight glutenin alleles in wheat. Euphytica 134: 51-60.

Miura H, Sugawara A. 1996. Dosage effects of the three *Wx* genes on amylose synthesis in wheat endosperm. Theor Appl Genet 93: 1066-1070.

Moczulski M, Salmanowicz BP. 2003. Multiplex PCR identification of wheat HMW glutenin subunit genes by allele-specific markers. J Appl Genet 44: 459-471.

Morita N, Maeda T, Miyazaki M, Yamamori M, Miura H, Ohtsuka I. 2002. Effect of substitution of waxy-wheat flour for common flour on dough and baking properties. Food Sci Technol Res 8: 119-124.

Nakamura T, Yamamori M, Hirano H, Hidaka S. 1993. Identification of three Wx proteins in wheat (*Triticum aestivum* L.). Bioch Genet 31: 75-86.

Nakamura T, Vrinten P, Saito M, Konda M. 2002. Rapid classification of partial waxy wheats using PCR-based markers. Genome 45: 1150-1156.

Payne PI, Holt LM, Law CN. 1981. Structural and genetical studies on the high-molecular-weight subunits of wheat glutenin. Part 1: Allelic variation in subunits amongst varieties of wheat (*Triticum aestivum*). Theor Appl Genet 60: 229-236.

Payne PI, Lawrence GJ. 1983. Catalogue of alleles for the complex gene loci, *Glu-A1*, *Glu-B1*, and *Glu-D1* which code for high molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res Commun 11: 29-35.

Shewry PR. 2003. Improving wheat quality: the role of biotechnology. In: Cauvain SP (ed.) Bread making - Improving quality. Woodhead Publishing Limited, Cambridge, UK, pp. 168-186.

Yamamori M, Quynh NT. 2000. Differential effects of *Wx-A1*, *-B1* and *-D1* protein deficiencies on apparent amylose content and starch pasting properties in common wheat. Theor Appl Genet 100: 32-38.

Wheat Inf. Serv. 103: 5-12, 2007. www.shigen.nig.ac.jp/ewis

Research Information

The study of stomatal characteristics in Iranian wheat wild accessions and land races

Shahram Mohammady*, Hamid Khazaei, Faiez Reisi
Faculty of Agriculture, University of Shahrekord, P.O. Box 115, Iran
*Corresponding author: Shahram Mohammady (E-mail: shfaza@hotmail.com)

Abstract
Wheat crops growing in arid and semi-arid regions generally experience some kinds of water-deficit. Water managing in the crop is partly depended on the stomatal characteristics. Therefore, reliable selection and breeding for suitable stomatal characteristics is of a great importance where water supplies are limited**.** This study was aimed to investigate different characteristics of stomata in Iranian wheat land races and wheat wild relatives not being studied so far. The results revealed that a huge variation exist between wheat land races and wheat wild relatives and even within the land races and wild relatives for different aspects of stomatal characteristics. Significant differences were found among the accessions of same ploidy level on the both adaxial and abaxial surfaces of flag leaf for stomatal frequency. Variation within diploid accessions was much higher than variation within either tetraploid or hexaploid wheats. The mean of stomatal frequency was the highest in diploids, intermediate in tetraploid and the least in hexaploids. Conversely, stomatal length and width was the lowest in diploids and highest in hexaploids indicating a negative relationship between stomatal frequency and stomatal size. Variations for stomatal area per unit leaf area were also found among and within poloidy levels. Nonetheless a particular trend was not found as the ploidy level changed. In general, this study revealed that wild species of wheat and landraces indicated variation for different aspects of stomatal characteristics and therefore they can be used in wheat breeding programs aiming to manipulate water-transpiration in wheat.

Introduction

Wheat is the first major world cereal and it has been cultivated within origin center of variation (southwest Asia) for over than 10,000 years. The wild species of wheat are still under cultivation in Iran, Iraq, east of Turkey, north of Palestine, Lebanon and Syria (Poehlman 1995). Braidwood et al. (1983), studying Zagros flanks of western Iran and northeastern Iraq reported that the cultivation of diploid and hexaploid wheats backed to 6500 years BC. Stomatal frequency and stomatal size have often been used as morphological markers for identifying ploidy level in many plant species, for example in *Acacia mearnsii* (Beck et al. 2003; 2005), *Actinidia deliciosa* (Przywara et al. 1989), *Aegilops neglecta* (Aryavand et al. 2003), *Bromus intermis* (Tan and Dunn 1973), *Coffea* L. (Mishra et al. 1991; 1997), *Dactylis* (Santen and Casler 1986), rye grass (Speckman et al. 1965), *Triticale* (Sapra et al. 1975), and *Triticum* spp. (Teare et al. 1971; Rajendra et al. 1978; Wang et al. 1989; Wang and Clarke 1993). Aryavand et al. (2003) reported that significant variation was found in stomatal frequency between the ploidy levels for basal leaves, the penultimate and flag leaves in *Aegilops negelecta* and stomatal frequency and size were highly negatively correlated. Selection and variation for stomatal characteristics has been reported in bread wheat (Bhgwat et al. 1993). In addition, stomatal characteristics have been also studied in wild species of other crops. It has been reported that stomatal size positively correlates with ploidy level and is negatively related to stomatal size in *Bromus inermis*, (Tan and Dunn 1975). Such a result has been also reported in *Coffea* L. (Mishra 1997).

Rajendra et al. (1978) reported that diploid, tetraploid and hexaploid wheats differ in stomatal frequency in which diploids having the highest and hexaploids the lowest number per unit area of leaf. Conversely, they reported that stomatal size increased with increasing ploidy level (2x<4x<6x). They also noticed that the adaxial flag leaf surface had a significantly higher stomatal frequency than the abaxial side.

Mohammady-D (2002) found significant differences for stomatal length in adaxial and abaxial surfaces of bread wheat leaves. However, he reported no differences between the adaxial and baxial surfaces of

leaves for stomatal width. He also reported that stomatal length is more effective than stomatal width on water transpiration. It is widely accepted that stomatal frequency is negatively correlated with stomatal size. Tanzarella and Blanco (1979) found that stomatal frequency of the flag leaf is negatively correlated with stomatal length in durum wheat.

Stomatal size and frequency have been used as an indicator of water loss by many investigators (Singh and Sethi 1995; Venora and Calcagno 1991; Wang and Clarke 1993a) but no published work was found to use stomatal pore width as a trait which determines the capacity of stomata to reduce water loss. This is possibly due to difficulties which appear during the measurement of stomatal pores especially under unfavorable conditions. Venora and Calcagno (1991) measured and used stomatal width as an indicator of stomatal aperture. This application seems to explain differences between the varieties for water loss only under non-limiting conditions provided varieties with larger size of stomata have larger stomatal pores. Wang and Clarke (1993b) reported that SF was positively correlated with the rate of water loss. This indicates breeding for smaller and fewer stomata may lead to reduction in water loss.

Much of the genetic variation for improving stress tolerance has been lost during selection and modern breeding (Araus et al., 2002). Therefore, other genetic materials such as landraces and wild species rather than modern varieties should be used to obtain a large improvement in stress tolerance. Selected landraces and wild species can contribute to the enhancement of wheat production in dry regions by direct use for cultivation or by using in various methods of plant breeding in order to improve high yielding but drought susceptible varieties so that they can tolerate water-stress. Finding variation in stomatal characterristics and indirect selection for water-stress tolerance using these characters are among subjects that are of interest for scientists. The objective of this study was to determine stomatal frequency, stomatal size (length and width) and stomatal area per unit leaf area in adaxial and abaxial surfaces of leaves in some Iranian land races and wild species not being investigated so far.

Materials and methods

Four diploid and 8 tetraploid accessions along with 5 hexaploid landraces and 3 cultivars, all except one collected from Iran, were used in this study. All the genotypes were provided by Cereal Research Department of Seed and Plant Improvement Institute, Iran. The vernalisation requirement for the genotypes and other descriptions were announced by the institute as cited in Table 1.

Seeds from accessions requiring vernalisation were germinated in petridishes and transferred into a growth chamber (2-4 $^{\circ}$C) for 5 weeks. Four weeks later other genotypes not sensitive to vernalisation were also germinated. Three seedlings from each accession were planted in plastic pots filled with 1.1 kg of a soil containing 42% sand, 36% silt and 22% clay. Each pot was brought to water-holding capacity by adding 250 ml of water in each pot. Pots were weighted every 3 day and amounts of water equal to the loss in weight were added (Ehdaie and Waines 1993). Eight days after transplanting the seedlings, two seedlings were removed from each pot leaving the most vigorous one. Pots were arranged in a randomized complete-block design with three replications in an unheated glasshouse at the University of Shahrekord, Iran. When flag leaves were fully developed, stomatal frequency and stomatal size (length and width) were measured on the adaxial and abaxial surfaces by impression method (Wang and Clarke 1993). Impressions were taken from the middle of the both adaxial and abaxial surfaces of flag leaves. The number of stomata was counted from seven different microscopic fields of view at 160X magnification. To find the stomatal Frequency (SF), the number of stomata per field of view was converted to the number of stomata per one mm^2 of leaf using a standard scale.

Stomatal length (SL) and stomatal width (SW) were measured on the both surfaces from the impressions using a scaled eyepiece of microscope and then stomatal size was converted to µm. Stomatal area per unit leaf area (SA) (µm of stomata/ mm^2 of leaf) was calculated using modified method of Wang and Clarke (1993) as a product of SF × SL × SW. The above measurements were made randomly on 20 stomata in each impression and the mean values of the 20 measurements were used for statistical analyses.

Data were analyzed using SAS, version 8.0 (copyright© 1999 by SAS Institute, Cary, NC, USA). Analyses of variance were preformed using the GLM procedure. Analyses of variance for diploid, tetraploid and hexaploid were also performed separately using non-orthogonal method. In addition, 3 contrasts, including 2x *vs.* 4x, 2x *vs.* 6x and 4x *vs.* 6x, were made in order to test variation between ploidy levels for the characters under study. Since none of the replications significantly affected the total variation, they were omitted from the tables of variance analyses. Correlation analysis was performed to determine the relationship between the traits using the CORR procedure and comparisons between means were made using LSD test.

Results

Stomatal frequency

Analysis of variance indicated that significant differences existed among the genotypes for stomatal

Table 1. Descriptions of the genotype used in the study

Accession	Ploidy level	Growth habit	Species	Origin
5172	2x	F	*T. monococcum*	Iran
5175	2x	W	*T. monococcum*	Iran
5196	2x	F	*T. monococcum*	Iran
3829	2x	W	*T. monococcum*	Iran
547	4x	F	*T. turgidum*	Iran
548	4x	F	*T. turgidum*	Iran
549	4x	F	*T. turgidum*	Iran
551	4x	F	*T. turgidum*	Iran
553	4x	F	*T. turgidum*	Iran
908	4x	F	*T. turgidum*	Iran
1551	4x	W	*T. turgidum*	Mosel, Iraq
1677	4x	F	*T. turgidum*	Iran
5074	6x	F	*T. aestivum*	Iran
5075	6x	W	*T. aestivum*	Iran
5243	6x	F	*T. aestivum*	Iran
5216	6x	S	*T. aestivum*	Iran
5068	6x	F	*T. aestivum*	Iran
Azar2	6x	F	*T. aestivum*	Iran
Sardari	6x	W	*T. aestivum*	Iran
Rooshan	6x	F	*T. aestivum*	Iran

F=Facultative, W=Winter, S=Spring

Table 2. Analysis of variance for stomatal frequency on the adaxial and abaxial surfaces of leaves

Source of variation	Degree of freedom	Mean square	
		adaxial	abaxial
Genotype	19	14080.5**	1504.4**
2x	3	493.1**	981.7
4x	7	227.1**	145.5
6x	7	113.6*	198.1
Error	38	40.1	670.2
2x *vs*. 4x	1	14204.3**	22339.0**
2x *vs*. 6x	1	22193.4**	22339.0**
4x *vs*. 6x	1	1331.4**	895.0**

*, **: significant at 5 and 1% of probability, respectively

frequency on the both adaxial and abaxial surfaces. However no significant difference was found among the accessions within ploidy levels for stomatal frequency on the abaxial surfaces (among the diploid, tetraploid and hexaploid accessions). The result of contrast between poloidy levels indicated that the variance between poloidy groups were highly significant (Table 2). All these significant variances imply that the genotypes used in the present study can be considered as a source of variation in selection for

Table 3. Analyses of variance for stomatal length (SL), stomatal width (SW), stomatal area per unit flag leaf area (SA) in the adaxial and abaxial surfaces

Source of variation	Degree of freedom	Mean square					
		adaxial			abaxial		
		SL	SW	SA	SL	SW	SA
Genotype	19	174.8**	47.7**	379.0**	129.1**	46.6**	185.0**
2x	3	69.0**	8.9*	115.0	15.0	12.5**	493.0**
4x	7	13.9*	6.3*	237.0**	23.6**	3.9	217.0**
6x	7	13.5*	9.8**	234.0**	22.3**	11.5**	67.5**
Error	38	6.0	1.5	60	5.5	2.5	17.0
2x *vs*. 4x	1	2331.5**	456.3**	3540.0**	1263.4**	361.8**	37.9
2x *vs*. 6x	1	2532.0**	749.2**	1795.0**	2032.4**	758.1**	25.0
4x *vs*. 6x	1	6.2	54.2**	462.0**	136.5**	108.8**	1.1

*,**: significant at 5% and 1% of probability, respectively.

the stomatal frequency.

Mean stomatal frequency of the adaxial and abaxial surfaces of accessions is presented in Table 4. The range of difference between genotypes was considerable in all ploidy groups. Stomatal frequency of the adaxial surface ranged from 94.86 to 125.26 for diploid, from 56.18 to 81.51 for the tetraploid and from 45.59 to 64.47 for the hexaploid accessions. For the abaxial surface, it varied from 79.60 to 111.90 for diploid, from 42.83 to 63.55 for tetraploid and from 34.08 to 52.04 for hexaploid accessions (Table 5).

According to the data shown in Table 4, the mean of stomatal frequency was the highest in diploids (108.45 and 96.01, for adaxial and abaxial surfaces, respectively), intermediate in tetraploid accessions (66.31 and 52.09 for adaxial and abaxial surfaces, respectively) and the lowest in hexaploid accessions (55.78 and 43.46 for adaxial and abaxial surfaces, respectively). It is also clear from the Table that the diploid accessions showed non overlapping range with the other ploidy levels for stomatal frequency for both adaxial and abaxial surfaces, but tetraploid and hexaploid accessions showed overlapping range on the both adaxial and abaxial surfaces. The latter subject implies that stomatal frequency could not be considered as a morphological marker for poloidy identification as it was earlier reported by Beck et al. (2003; 2005) in *Acacia mearnsii*, Przywara et al. (1989) in *Actinidia deliciosa*, Aryavand et al. (2003) in *Aegilops neglecta* and Tan and Dunn (1973) in *Bromus intermis*. From the present study, in general, it can be deduced that stomatal frequency decrease with increasing in ploidy level. The similar results have been also reported by Teare et al. (1971), Rajendra et al. (1978), Wang and Clarke (1993) in wheat, Mishra (1997) in *coffea* and Aryavand et al. (2003) in *Aegilops*.

For all accessions except 3829 the adaxial surface had higher stomatal frequency than the abaxial surface. Nonetheless, the differences between the 2 sides were not significant ($t = 1.88^{ns}$, $df = 38$). Despite the present results, other studies have reported significant differences between adaxial and abaxial surfaces in wheat (Teare et al. 1971; Rajendra et al. 1978; Singht and Sethi 1995; Mohammady-D 2002).

The ratio of stomatal frequency on abaxial to adaxial surface was 0.81 over all studied genotypes. Mohammady-D (2002) obtained this ratio 0.69 for *T. aestivum*, and suggested that the ratio is possibly more stable that the absolute number of stomata in either surfaces of flag leaf. A highly positive correlation was observed between adaxial and abaxial surfaces for stomatal frequency ($r = 0.915^{**}$). This indicates that selection based on analysis on one side of leaves is sufficient and there is no need to measure stomatal frequency on the both sides.

Stomatal length

Analysis of variance indicated significant differences for stomatal length existed among accessions and also among diploids and hexaploids on the adaxial surface and among diploid and tetraploid accessions in the abaxial surface (Table 3). The contrast analysis indicated significant difference for stomatal length existed between diploids and tetraploids, diploids and hexaploids and between tetraploids and hexaploids on the abaxial surface. A similar trend was observed for the character on the adaxial surface except that no significant difference observed between tetraploids and hexaploids (Table 3).

Mean stomatal length of the adaxial surface are presented in Table 4. Stomatal length of the adaxial surface ranged from 26.17 to 37.88 µm for diploid

Table 4. Comparison of means for stomatal frequency (SF), stomatal length (SL), stomatal width (SW) and stomatal area per unit leaf area (SA) on the adaxial surface

Accession No.	Ploidy level	SF	SL	SW	SA
5172	2x	103.62	31.63	17.42	56739.00
5175	2x	125.26	26.17	20.84	68292.00
5196	2x	94.86	37.88	19.17	68689.00
3829	2x	94.86	37.88	19.17	68689.00
547	4x	58.48	51.00	28.21	83484.00
548	4x	56.18	49.67	27.04	75746.00
549	4x	73.68	47.84	24.54	86206.00
551	4x	58.48	51.67	24.59	73530.00
553	4x	66.77	49.17	25.46	83533.00
908	4x	64.93	47.46	26.25	80872.00
1551	4x	70.46	49.05	25.38	87594.00
1677	4x	81.51	44.84	28.05	102691.00
5074	6x	55.72	49.25	25.80	70832.00
5075	6x	45.59	50.63	29.17	67570.00
5243	6x	63.55	49.80	26.08	81996.00
5216	6x	54.80	47.71	29.21	76031.00
5068	6x	64.47	51.63	28.42	94618.00
Azar2	6x	53.88	50.09	31.13	83818.00
Sardari	6x	51.58	51.88	29.37	78711.00
Rooshan	6x	56.65	45.46	27.34	70425.00
$LSD_{0.05}$	-	10.55	4.02	2.00	12820

accessions, from 44.84 to 51.67 µm for tetraploid accessions and from 45.46 to 51.88 µm for hexaploid accessions (Table 4). For the abaxial surface (Table 5), it varied from 29.37 to 34.29 µm for diploid accessions, from 40.95 to 48.91 µm for tetraploid accessions and from 42.92 to 51.54 µm for hexaploid accessions.

The mean of stomatal length in diploid accession was the smallest (31.76 and 31.20 µm, for adaxial and abaxial surfaces, respectively) and it increased more or less with increasing ploidy level. These observations are similar to those reported by Rajendra et al. (1978), in *Triticum*, Borrino and Powel (1988) in *Hurdeum*, Przywara et al. (1988) in *Actinida*, Singh and Sethi (1995) in *Triticale* and Beck et al. (2003; 2005) in *Acacia*. The diploid accessions showed non-overlapping range with other ploidy level in both leaf sides, but tetraploid and hexaploid accessions had overlapping range. The adaxial surface had a higher stomatal length than abaxial surface in all accessions except 5172 (Tables 4 and 5). However, the differences were not significant based on t-test ($t = 1.38^{ns}$, df = 38).

Stomatal width

Significant differences were found for stomatal width among accessions, among diploid and tetraploid accessions for the adaxial and abaxial surfaces and among hexaploid accessions only in adaxial surface, but not among hexaploid accessions in abaxial surface (Table 3).

Table 5. Comparison of means for stomatal frequency (SF), stomatal length (SL), stomatal width (SW) and stomatal area per unit leaf area (SA) on the abaxial surface

Accession No.	Ploidy level	SF	SL	SW	SA
5172	2x	79.60	34.29	16.50	45010.00
5175	2x	111.90	29.37	18.24	60562.00
5196	2x	81.05	31.37	16.71	42340.00
3829	2x	111.44	29.75	20.92	69298.00
547	4x	52.50	40.95	25.87	55625.00
548	4x	42.83	48.91	26.92	56318.00
549	4x	50.20	42.79	24.58	52697.00
551	4x	50.65	42.00	23.21	49101.00
553	4x	51.58	42.83	23.96	52625.00
908	4x	60.33	41.87	24.37	61542.00
1551	4x	45.13	43.50	24.96	48900.00
1677	4x	63.55	47.25	25.00	74916.00
5074	6x	53.42	44.88	24.50	58618.00
5075	6x	44.67	45.62	26.88	54594.00
5243	6x	38.22	47.71	29.25	53401.00
5216	6x	52.04	46.46	27.42	65885.00
5068	6x	38.22	49.00	29.16	54574.00
Azar2	6x	35.00	48.96	30.17	51635.00
Sardari	6x	34.08	51.54	29.42	51651.00
Rooshan	6x	52.04	42.92	26.17	58272.00
$LSD_{0.05}$	-	6.94	3.87	2.62	6937.00

The contrast analysis indicated significant difference existed between diploid *vs*. hexaploid accessions and tetraploid *vs*. hexaploid accessions for the both surfaces. Similar to stomatal length, mean of stomatal width increased with increasing in ploidy level. Nonetheless, the similar data in diploid, tetraploid and hexaploid genotypes for SW indicates that this character is not a suitable marker for distinguishing between ploidy levels.

Stomatal area per unit leaf area (SA)

For stomatal area per unit leaf area significant differences were found among accessions and within diploids and hexaploids for both surfaces and within tetraploid group for the abaxial surface only (Table 3). Contrast analysis indicated significant difference between ploidy levels for the adaxial surface. Tetraploid accessions had highest stomatal area per unit leaf area (mean 84207.12), and hexaploids had intermediate amount (mean 78000.35) and finally diploid accessions had the lowest amount of SA (63170.69) (Table 4).

Discussion

It is theoretically expected that varieties with higher number of stomata per unit area and greater length and width of stomata lose more water during the growth period. This happens if stomata remain open during the water-stress period. Thus, any response of stomata

to water-stress can be discussed in relation to stomatal resistance and Leaf Relative Water Content (LRWC). Reduction in water loss from leaf surfaces during periods of severe water-stress is an important drought tolerance indicator. Low rate of cuticle transpiration, therefore, may reduce leaf dehydration and promote leaf survival (Wang and Clarke 1993b). When water-stress develops, the response of stomata to water-stress seems to be of a great importance in reducing water loss comparing with stomatal characteristics. Mohammady-D (2002) studied the relationships between stomatal characteristics and water status in 2 wheat varieties including Falchetto (water-stress tolerance) and Oxley (water-stress susceptible). He reported that stomatal frequency of Falchetto was significantly higher than Oxley, but Falchetto had smaller stomata. On the other hand, his results revealed that Falchetto had a higher Leaf Relative Water Content (LRWC) and Stomatal Resistance (SR) than Oxley. These results indicated that SF is not always correlated with plant water status. This is because stomatal size, response of stomata to environmental stress and even cuticle resistance are also involved in determining plant water status particularly under water-stress conditions. In the case of cuticle resistance, Gupta *et al.* (2001) using diurnal observations, reported that a drought tolerant variety (C-306) had higher leaf diffusive resistance than a drought sensitive variety (Kalyansona). Since stomata are mostly closed at night, it can be concluded that differences in diffusive resistance are mostly due to differences in cuticle resistance.

The results of other workers concerning the relationship between stomatal characteristics and plant water status are inconsistent. McCaig and Romagosa (1989) found no consistent differences in SF between two durum genotypes with different water-retention capabilities. Wang and Clarke (1993b) reported that SF was not correlated with relative water loss and LRWC in field experiments. However, their results indicated that SF was positively correlated with the rate of water loss but not with LRWC under growth room experiments. The inconsistency of this relationship is possibly due to the influence of other characteristics of stomata rather than SF and due to negative relationships between stomatal size and frequency. In addition to SF and stomatal size, the stomatal responses to water-stress and cuticle resistance are other factors which influence water status of plants under water-stress conditions. Thus, the results presented in this section and those explained above indicate that stomatal characteristics are affecting water status of plants as a complex, and every component of this complex should be studied in relation to other components and with other factors which influence water status of plants.

The increase or decrease in transpiring area under stress conditions may not be achieved by selecting for high or low SF due to the negative correlation between SF and stomatal size (Venora and Calcagno 1991). For this reason, it seems that SA as a combination of SF, SL and SW is a better determination of water status in plants. The relationship between stomatal resistance and stomatal characteristics is also important in determining water status of crops under water-stress conditions. In a study carried out by Mohammady-D (2002), SR and SA were investigated in Varieties Falchetto (water-stress resistant) and Oxley (water-stress susceptible). He reported no significant differences between the two varieties for SA on the both surfaces of leaves but highly significant difference for stomatal resistance was observed between the two varieties. These results indicate that higher SR of Falchetto on the adaxial surface is not due to smaller SA but is possibly due to either differences in stomatal response to water-stress or differences in cuticle resistance.

The most important issue regarding water-stress tolerance is that the characteristics of stomata of the crop must match the pattern of water supply (Passioura 1996). When the water supply is insufficient from the onset of growth, less number of stomata and low stomatal area can lead to a conservative consumption of water and thus can be considered as a suitable adaptive trait. On the other side, when there is a small shortage of water supply happens at the end of growth cycle, low stomatal frequency or area, or even low stomatal transpiration, have no benefit to the crops due to low photosynthesis and thus enhancing yield reduction in crops.

In general, wild species of wheat and landraces used in the present study indicated variation for different aspects of stomatal characteristics and therefore they can be used in wheat breeding programs aiming to manipulate stomatal characteristics. There is also a need to evaluate these genotypes for other traits related to water statue in order to come to a conclusion about their promising for being involved in wheat breeding programs aiming to improve wheat cultivars for water statues in particular in dry regions.

References

Araus JL, Slafer GA, Reynolds MP, Royo C. 2002. Plant breeding and drought in C-3 cereals: What should we breed for? Annals of Bot 89: 925-940.

Aryavand A, Ehdaie B, Tran B, Waines JD. 2003. Stomatal frequency and size differentiate ploidy level in *Aegilops neglecta*. Genet Resources Crop Evol 50: 175-182.

Beck SL, Dunlop RW, Fossey A. 2003. Stomatal length and frequency as a measure of ploidy level in black wattle, *Acacia mearnsii* (de Wild). Bot J Linnean Society 141: 177-181.

Beck SL, Visser G, Dunlop RW. 2005. A comparison of direct (flow cytometry) and indirect (stomatal guard cell lengths and chloroplast numbers) techniques as a measure of ploidy level in black

wattle, *Acacia mearnsii* (de Wild). South African J Bot 71: 354-358.
Bhagwat SG, Bhatia CR. 1993. Selection for flag leaf stomatal frequency in bread wheat. Plant Breed 110: 129-136.
Borrino EM, Powell W. 1988. Stomatal guard cell length as an indicator of ploidy in microspore plants of barley. Genome 30: 158-160.
Braidwood LS, Braidwood, JR, Howe B, Reed CA, Watson PJ. 1983. Prehistoric archeology along the Zagros Flanks. Oriental Institute Publication, Chicago, USA.
Ehdaie B, Waines JG. 1993. Variation in water use efficiency and its components in wheat: I. well water pot experiment. Crop Sci 33: 294-299.
Gupta NK, Gupta S, Kumar A. 2001. Effect of water stress on physiological attributes and their relationship with growth and yield of wheat cultivars at different stages. J Agr Crop Sci 186: 55-62.
McCaig TN, Romagosa I. 1989. Measurement and use of excised-leaf water status in wheat. Crop Sci 29: 1140-1145.
Mishra MK. 1997. Stomatal Characteristics at different ploidy levels in *Coffea* L. Annals of Bot 80: 689-692.
Mishra MK, Prakash NS, Sreenivasan MS. 1991. Relationship of stomatal length and frequency to ploidy level *Coffea* L. J Coffee Res 21: 32-41.
Mohammady-D S. 2002. Inheritance of tolerance to water stress in wheat (*Triticum aestivum*). Ph D Thesis. University of Newcastle upon Tyne, UK.
Passioura JB. 1996. Drought and drought tolerance. Plant Growth Regulation 20: 79-83.
Poehlman JM. 1995. Breeding Field Crops. Iowa State University Press, Iowa, USA.
Przywara L, Pandeyf K.K, Sanders PS. 1988. Length of stomata as an indicator of ploidy level in *Actinidia deliciosa*. New Zealand J Bot 26: 179-182.
Rajendra BR, Mujeeb KA, Bates LS. 1978. Relationships between 2x *Hordum* sp., 2x *Secale* sp. And 2x, 4x, 6x *Triticum* sp. for stomatal frequency, size and distribution. Environ Exp Bot 18: 33-37.
Santen EV, Casler EV. 1986. Evaluation of indirect ploidy indicators in *Dactylis* C. Sub species. Crop Sci 26: 848-852.
Sapra VT, Hughes JL, Sharma GC. 1975. Frequency, size and distribution of stomata in Triticale leaves. Crop Sci 15: 356-358.
Singh S, Sethi GS. 1995. Stomatal size, frequency and distribution in *Triticum aestivum*, *Secale cereale* and their amphiploids. Cereal Res Commun 23: 103-108.
Speckman G, Post J, Dijkstra H. 1965. The length of stomata as an indicator for polyploidy in rye grasses. Euphytica 14: 225-230.
Tan GY, Dunn GM. 1975. Stomatal length, frequency and distribution in *Bromus inermis* Leyss. Crop Sci 15: 283–286.
Tanzarella OA, Blanco A. 1979. Stomatal frequency and size in durum wheat. Genet Agri 33: 355-362.
Teare ID, Peterson CJ, Law AG. 1971. Size and frequency of leaf stomata in cultivars of *Triticum aestivum* and other *Triticum* species. Crop Sci 11: 496-498.
Venora G, Calcagno F. 1991 Study of stomatal parameters for selection of drought resistant varieties in *Triticum durum* Desf. Euphytica 57: 275-283.
Wang H, Clarke JM. 1993a. Genotypic, intraplant, and environmental variation in stomatal frequency and size in wheat. Canadian J Plant Sci 73: 671-678.
Wang H, Clarke JM. 1993b. Relationship of excised-leaf water-loss and stomatal frequency in wheat. Canadian J Plant Sci 73: 93-99.
Wang PY, Chen R, Fang R, Zhu Z. 1989. Study of identification of ploidy in pollen plants and wheat using guard cells. Acta Agricultural Universities Pekinensis 15: 141-145.

Wheat Inf. Serv. 103: 13-14, 2007. www.shigen.nig.ac.jp/ewis

Others

Instructions to Authors

eWIS welcomes manuscripts that provide test results, technical tips, protocols, mutant and germplasm descriptions, map information, and any other information that may be useful in the lab and field. The articles are informal, non-peer-reviewed, thus do not constitute formal publications. Only manuscripts that require minimal editing will be considered for publication.

Note well that the data and ideas published in eWIS are made available by their authors with the understanding that they will not be used in publications without the authors' specific consent. This means that the eWIS articles may not be cited without permission from the authors. Copyright is retained by the author.

Manuscript Submission

Only manuscripts written in English will be considered for publication. Follow the Instructions strictly. Refer to the latest eWIS articles for the format.

Electronic submission is mandatory. **All manuscripts should be submitted using the online manuscript submission system** in the eWIS page (**http://shigen.lab.nig.ac.jp/ewis/**) that is linked from the "KOMUGI" page (http://shigen.lab.nig.ac.jp/wheat/komugi/top/top.jsp/).

Editorial Office will inform authors of the status of their manuscript via e-mail as quickly as possible. The "eWIS online submission system" offers easy and straightforward web-based submission procedures. For text writing, Microsoft Word is recommended. Manuscripts should be double-spaced and page-numbered starting from the title page. Do not use line numbers. Figures including illustrations, photographs and color plates should be submitted as JPEG files. PDF is not an acceptable file format.

Manuscript Categories

eWIS accepts the following categories of papers:

(1) **Research information**: Original research articles in the field of wheat sciences

The manuscript should start with a title, the names of author(s), affiliation(s), abstract, followed by the text. Abstract may be omitted if not necessary. There is no fixed limit on the length but a concise presentation is encouraged.

(2) **Research Opinion & Topics**: Reviews, mini-reviews, trends and topics in wheat research.

Authors who wish to submit a (mini-)review should contact the Editorial Office prior to submission.

(3) **Meeting Reports**: Announcement of forthcoming meeting and reports on the meeting attended

(4) **Others**: Any other information useful for wheat researchers

Title, Affiliation and Abstract

In the title page(s), the manuscript category (as mentioned above), a title, the names of the author(s), affiliation(s) and address(es) of the authors, and the e-mail address, telephone, and fax numbers of the corresponding author must be clearly indicated.

The Abstract (100-250 words) may not contain references.

References

References should be cited in the text by the author(s) and year, and listed at the end of the text with the names of authors arranged alphabetically. When an article has more than two authors, only the first author's name should appear, followed by "et al.", in the text. The references should be formatted as follows.

Journal articles:

Payne PI, Holt LM, Law CN (1981) Structural and genetical studies on the high molecular weight subunits of wheat glutenin. Theor Appl Genet 60:229-236.

Book chapters:

Peacock WJ, Dennis ES, Gerlach WJ (1981) Molecular aspects of wheat evolution: repeated DNA sequences. In: Evans LT and Peacock WJ (eds.) Wheat Science - Today and Tomorrow. Cambridge Univ. Press, Cambridge, UK, pp. 41-60.

Books:

Knott DR (1989) The Wheat Rusts - Breeding for Rust Resistance. Springer-Verlag, New York, USA.

Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., K. Tsunewaki personal communication).

Abbreviations
Abbreviations should be explained at first occurrence.

Symbols and Units
Gene names and protein names must carefully be discriminated. Gene names and loci should be italicized; protein should be upright. The SI units (http://physics.nist.gov/Pubs/SP330/contents.html) should be used throughout.

Nomenclature
Nomenclature of genes and chromosomes should follow the 'Catalogue of gene symbols for wheat' (McIntosh *et al.*: 10th Int. Wheat Genet. Symp. 2003).

Nucleotide sequences
The DDBJ/EMBL/GenBank accession numbers must be provided for newly reported nucleotide sequences.

Tables
Tables must be numbered consecutively. For Table writing, Microsoft Word is recommended. Prepare a separate file for each table. Refer to the latest eWIS articles for format.

Figures
Figures must be numbered consecutively. Prepare a separate file for each figure.

Outline of the publication process
Authors of accepted manuscripts are informed by e-mail that a temporary URL has been created from which they can obtain their proof. Proofreading is the responsibility of the author. Authors should make proof corrections and send them to Editorial Office by e-mail. After online publication, corrections can only be made in exceptional cases when Editorial Office permits the necessity.

The final version of accepted manuscripts will be published in the 'Online First' section of the eWIS web page upon receipt of proof corrections. Editorial Office biannually gathers the accepted manuscripts published in the 'Online First' into a volume. In 'Archive' of eWIS, all manuscripts are collected as PDF format, and open to all wheat researchers.

No hard-copy edition will be supplied. For each volume, a PDF edition will be available for downloading.

Wheat Information Service No. 103

---

1 March 2007
www.shigen.nig.ac.jp/ewis