Stomatal Variation in Wheat-*Thinopyrum elongatum* Disomic Addition Lines

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Abstract

Stomatal characteristics are inconsistent and greatly influenced by genetics of the plant and environmental conditions. Present study aimed to determine the impact of addition of a pair of chromosomes from Thinopyrum elongatum (2n=14, Genome EE) to common wheat (Triticum aestivum cv. "Chinese Spring"; 2n=42, Genome AABBDD) on stomatal characteristics. Altogether, seven Wheat-Th. elongatum disomic addition lines and a control (Chinese Spring) were used to characterize the density, length, and width of stomata, and the total stomatal area in normal and flag leaves by using leaf impression method. The leaf impressions were made from the middle of the leaves of different wheat lines. The leaves used for impression cast were of the same age. Stomatal density was measured in terms of number of stomata under the field of vision at 400X magnification, while the size (length and width) measurements of individual stomata were done by using ImageJ software. With the exception of flag leaves of 1E disomic addition line, the total stomatal area in leaves of all the lines were significantly lower than that in the control, irrespective of leaf types (normal or flag). These results indicate the potential role of additional chrromosomes of Th. elongatum in stress tolerance in wheat.

Keywords: Chinese spring, density, stomata, addition line

Introduction

Stomata are small apertures found on both adaxial and abaxial sides of the leaf surfaces meant for regulation of photosynthetic CO_2 uptake and transpiration. The number, size and distribution of stomata depend upon environmental condition: for example plants grown under conditions of high light intensity and lower level of CO_2 show higher stomatal

density (Petrova, 2012; Woodward and Kelly 1995). Similarly, stomatal traits are also controlled genetically as proved in *Arabidopsis,* where at least 40 genes are known to contribute for the stomatal development (Pillitteri and Torii, 2012). The size (length) of stomata ranges from 10 micrometer to 80 micrometer with densities ranging from 5 to 100 stomata per square millimeter. There is a negative relationship between stomata size and density in all plants i.e. larger stomata are found in low density. It is estimated that total stomatal pore area constitutes 5% of the leaf surface but is responsible for loss of 70% of total water used by plants (Hetherington and Woodward, 2003). Therefore, knowing genetic control and molecular mechanisms of stomata characteristics plays a significant role in developing drought tolerant plants. During drought condition plant stomata must open to allow uptake of CO_2 and after CO_2 intake it must be closed to minimize the transpiration (Geber and Dwson, 1990). The size, number, and distribution of stomata on the surface of leaf affects transpiration and gas exchange rates (Ciha and Brun, 1975).

Flag leaf plays an important role in carbohydrate synthesis, accumulation and portioning of the photosynthates during the grain filling period, and affects grain yield under normal as well as drought conditions (Biswal and Kohli, 2013). When the stomata restrict water loss from the flag leaf during drought condition the plant will survive longer but premature closure of stomata during drought reduces photosynthesis (Biswal and Kohli, 2013).

The number of grains per spike plays a significant role in final yield during harvesting. Total grain weight per plant directly reveals the systematic use of nutrients and their translocation into generative parts of plant (Borojevic, 1983). Number of seeds per spike is influenced by several factors such as varieties, translocation of assimilates from leaves and stem to grain, farming and soil conditions and senescence period. In hybrid rice, the plants having leaves with large sized and dense stomata produced longer panicle, higher number of filled grains per panicle and heavier grains (Sarwar *et al*., 2013). In the present study various stomatal traits like distribution, density and size of stomata are compared among different Wheat-*Th. elongatum* disomic addition lines and a normal Chinese spring as control.

Methods

Plant material

Seven addition lines of common wheat cv. 'Chinese Spring' (CS) were used. The Wheat-*Thinopyrum elongatum* disomic addition lines (CS+1E", CS+2E", CS+3E", CS+4E", CS+5E", CS+6E" and CS+7E") were produced by Dvorak (1980). Normal CS possesses 42

chromosomes while each disomic addition line possesses a pair of respective chromosomes (EE Chromosome) along with normal wheat chromosomes. These lines were obtained from National Bio Resource Project-Wheat, (NBRP-Wheat) Japan (http://www.shigen.nig.ac.jp/ wheat /komugi/top/top.jsp).

Stomatal analysis

Seven Wheat-*Thinopyrum elongatum* disomic addition lines were selected for the stomata analysis. The analysis was carried out using two months old normal leaves and fully expanded mature flag leaves selected from the potted plants. They were cleaned in tap water and dried. The upper and lower surfaces of the leaves were then carefully polished with nail varnish for 10 minutes. Then the dried nail varnish was peeled out using transparent tape, mounted on slide and observed under microscope (LABOMED INC. Los Angeles CA. USA). Six different observations were taken from three slide of each plant line and mean was calculated to find out stomata density per microscopic field (400X magnification). Similarly, length and width of stomata was measured by ImageJ software and mean was calculated for each wheat line. Stomatal area for each leaf surface was calculated by multiplying the density, length and width of the stomata. The total stomatal area was calculated by adding the stomatal areas of two surfaces of respective leaves.

Statistical Analysis

All measurements were done six times. Statistical analysis was done by using Microsoft excel and R software. The statistical significance of differences in stomata density and size were analyzed using ANOVA. The box plot diagram on variation in total stomatal area in different wheat lines were prepared by using R open source software.

Results

Stomatal density

The image and data of stomatal density on upper and lower surfaces of young normal leaf and that of mature flag leaf is shown in Fig. 1 and Table 1 respectively. In the case of normal leaves the highest stomatal density on both the upper (39.6±3.0) and lower surface (31.3±3.4) and lower surface) was observed in CS+5E'' line. At the same time the lowest stomatal density in normal leaves was found in CS+4E'' for upper surface (21.1±2.3) and in CS+3E'' for lower surface (11.5±1.6). In the case of flag leaves the highest density of stomata on upper surface (56.0±5.3) was observed in CS+5E'' while that on lower surface (41.5±3.3) was observed in CS+1E''. The lowest stomatal density in flag leaves was found in CS+7E'' for upper surface (42.1±25.5) and in CS+3E'' for lower surface (17.0±2.0). With the exception of CS+5E'', the stomatal density in all the addition lines was found to be lower

than in 'Chinese Spring' (control), irrespective of the leaf type and surface. The stomatal density in most of the addition lines were significantly different (P<0.05) than in control.

Table 1: Stomata density in normal and flag leaves of different wheat lines

Wheat Line	Stomatal density			
	Normal leaf		Flag Leaf	
	Upper surface	Lower surface	Upper surface	Lower surface
CS+1E"	34.8±4.0[cd]	18.0±3.5 [bc]	50.0±1.5 [abc]	41.5±3.3 [d]
CS+2E"	28.5±1.5 [b]	15.5±2.5 [abc]	45.5±2.7 [ab]	25.6±3.9[bc]
CS+3E"	26.8±1.7 [ab]	11.5±1.6 [a]	50.3±4.8 [abc]	17.0±2.0 [a]
CS+4E"	21.1±2.3 [a]	13.3±1.9 [ab]	50.8±2.6[bc]	30.5±4.4 [c]
CS+5E"	39.6±3.0 [d]	31.3±3.4 [d]	56.0±5.3 [c]	31.3±5.1 [c]
CS+6E"	30.0±2.8 [bc]	16.8±3.7 [bc]	45.3±7.0[ab]	22.0±3.5 [ab]
CS+7E"	25.8±5.9 [ab]	13.3±2.5 [ab]	42.1±5.5 [a]	29.1±1.7 [c]
CS	31.1±3.6 [bc]	19.0±2.3 [c]	49.1±3.7 [abc]	38.6±4.5 [d]

Note: In each column the values with different letters are significantly different (P<0.05)
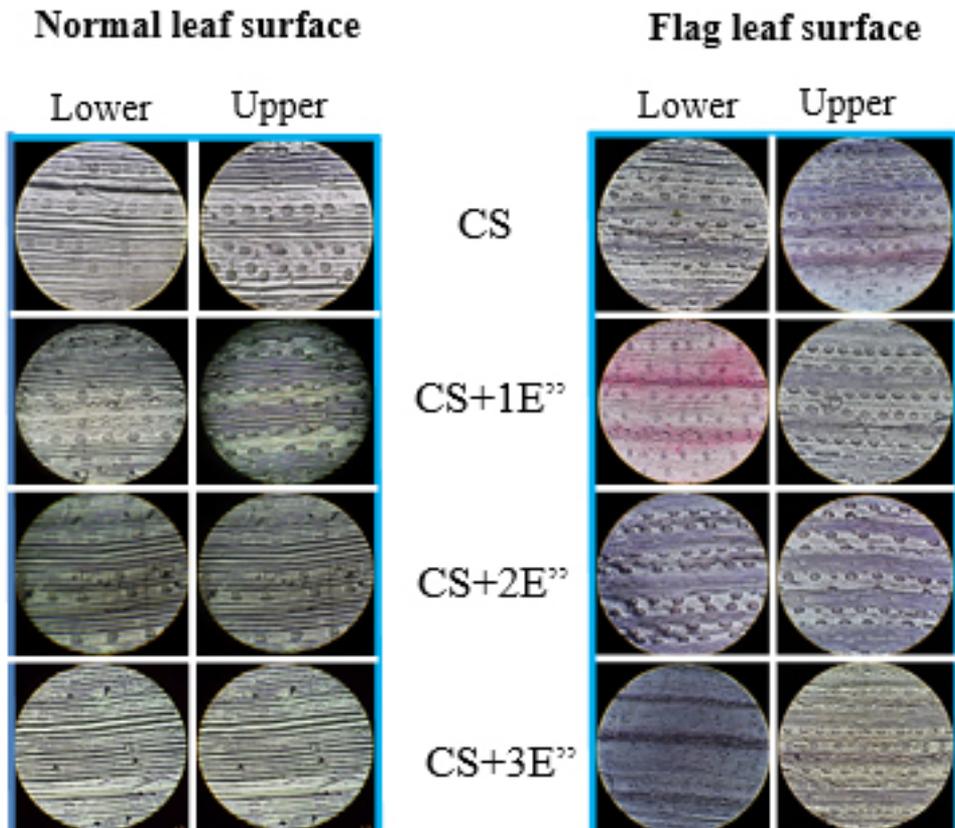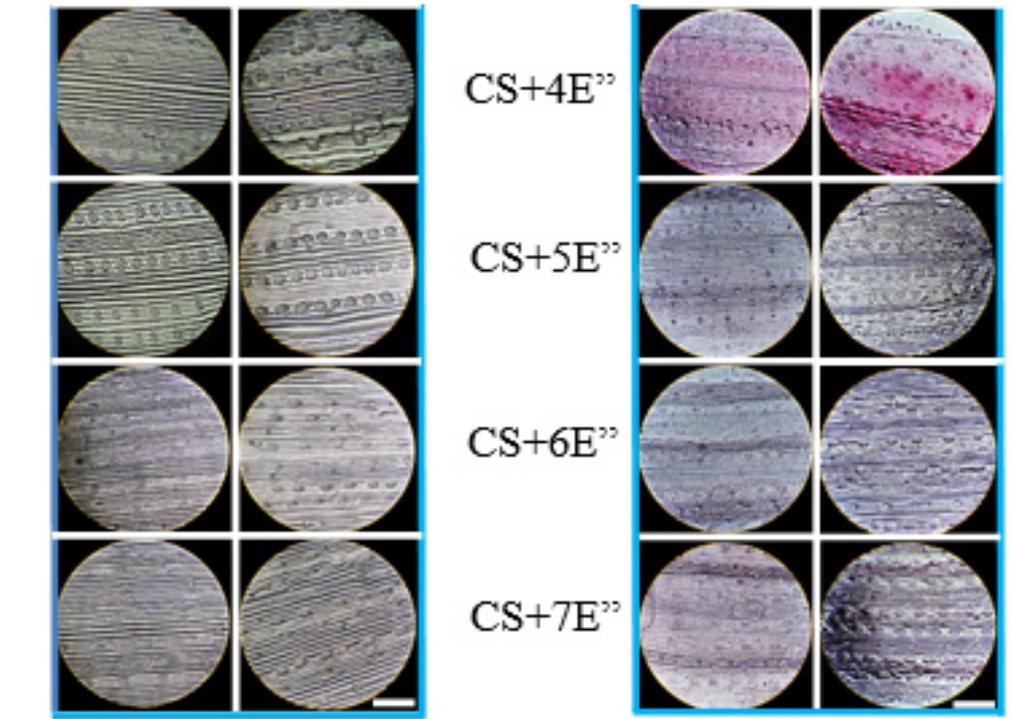
Figure 1. Microscopic image of leaf impression showing distribution of stomata on both surfaces of Normal and Flag leaves of Wheat cv. Chinese Spring (CS) and seven Wheat-Th. elongatum disomic addition lines (CS+1E" to CS+7E").

Note: CS: Chinese Spring wheat; 1E": a pair of *Th. elongatum* 1E chromosome in CS genetic background; Bar (white at lower right image) = 0.1mm.

Length of Stomata

The variation in the length of stomata from upper and lower surfaces of normal and flag leaves in different wheat lines is presented in Table 2. In the case of normal leaves, the length of stomata on upper surface was significantly longest in the case of Chinese spring (16.6±2.0 µm) and shortest in the case of CS+4E" (13.0±2.3 µm). Similarly, the length of stomata on the lower surface of normal leaf was found to be longest in CS+1E" (14.5±1.2 µm) and shortest in CS+6E" (9.60±1.5 µm). In the case of flag leaves the length of the stomata on upper surface was longest (29.3±2.8 µm) in Chinese spring and shortest (21.8±1.7 µm) in CS+5E" while that on lower surface was longest (26.3±2.1 µm) in CS+7E" and shortest (17.1±1.3 µm) in CS+5E".

Table 2: Length of Stomata in normal and flag leaves of different wheat lines

Wheat Line	Stomatal Length(μm)			
	Normal leaf		Flag Leaf	
	Upper surface	Lower surface	Upper surface	Lower surface
CS+1E"	16.3±1.6 [bc]	14.5±1.2 [b]	26.0±2.3 [bcd]	19.0±1.6 [ab]
CS+2E"	15.5±0.8 [bc]	14.0±1.1 [b]	26.8±2.4 [cd]	17.8±1.8 [a]
CS+3E"	15.6±1.0 [abc]	13.0±0.6 [b]	23.0±1.6 [ab]	19.0±2.0 [ab]
CS+4E"	13.0±2.3 [a]	12.6±1.2 [b]	23.0±2.4 [ab]	21.0±2.0 [ab]
CS+5E"	13.8±1.47 [abc]	12.0±1.7 [ab]	21.8±1.7 [a]	17.1±1.3 [a]
CS+6E"	14.1±1.1 [abc]	9.60±1.5 [a]	23.6±1.0 [abc]	19.8±2.4 [ab]
CS+7E"	13.5±1.8 [ab]	12.1±1.4 [ab]	28.3±1.0 [d]	26.3±2.1 [c]
CS	16.6±2.0 [c]	13.0±1.7 [b]	29.3±2.8 [d]	22.1±2.8 [b]

Note: In each column values with different letters are significantly different (P<0.05) within the group.

Width of Stomata

The variation in the width of stomata from upper and lower surfaces of normal and flag leaves in different wheat lines is presented in Table 3. In the case of normal leaves, the stomata on upper surface were widest in Chinese spring (28.5±2.3 μm) and narrowest in CS+4E" (16.8±1.3 μm). Similarly, the stomata on the lower surface of the normal leaves were also widest in Chinese spring (29.0±5.7 μm) and narrowest in CS+5E" (15.1±0.4 μm). In the case of flag leaves, the widest stomata on upper surface (16.6±2.0 μm) and lower surface (14.5±1.2 μm) were found in CS+4E". Chinese Spring and CS+1E", respectively. The narrowest stomata in the flag leaves however, were found in CS+4E" (13.0±2.3 μm) and CS+6E" (9.67±1.5 μm), on upper and lower surface, respectively.

Table 3. Width of stomata in normal and flag leaves in different wheat lines

Wheat Line	Stomatal Width (μm)			
	Normal leaf		Flag Leaf	
	Upper surface	Lower surface	Upper surface	Lower surface
CS+1E"	16.8±1.3 [a]	19.1±1.1 [ab]	16.3±1.6 [a]	14.5±1.2 [b]
CS+2E"	18.6±1.6 [a]	18.8±0.4 [ab]	15.5±0.8 [ab]	14.0±1.1 [b]
CS+3E"	19.1±0.7 [a]	18.0±1.9 [a]	15.6±1.0 [bc]	13.0±0.6 [b]
CS+4E"	18.8±1.7 [a]	18.6±1.0 [ab]	13.0±2.3 [ab]	12.6±1.2 [b]
CS+5E"	23.3±0.8 [b]	15.1±0.4 [a]	13.8±1.4 [c]	12.0±1.7 [ab]
CS+6E"	27.3±1.6 [c]	22.3±1.5 [b]	14.1±1.1 [d]	9.67±1.5 [a]
CS+7E"	18.0±1.1 [a]	17.8±1.4 [a]	13.5±1.8 [e]	12.1±1.4 [ab]
CS	28.5±2.3 [c]	29.0±5.7 [c]	16.6±2.0 [e]	13.0±1.7 [b]

Note: In each column values with different letters are significantly different (P<0.05) within the group

Total stomatal area
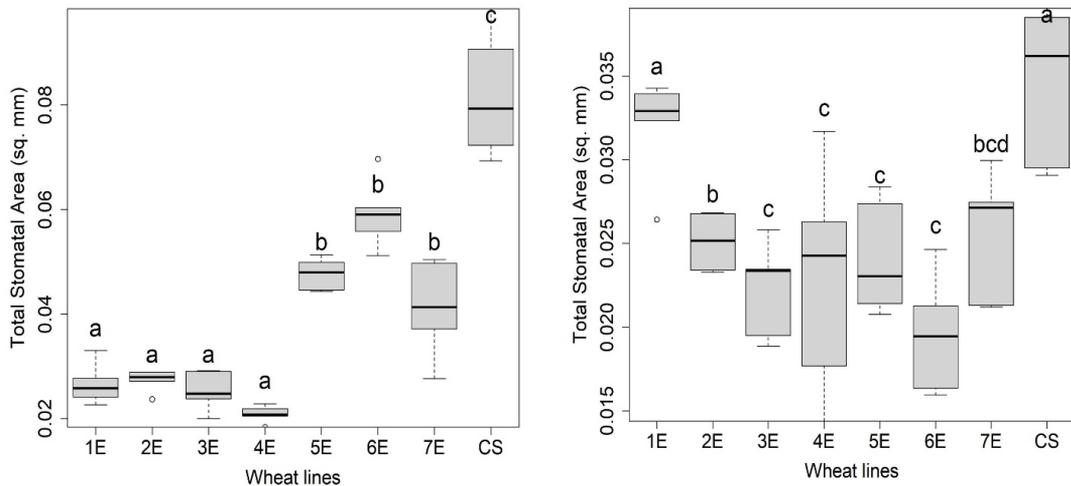
Figure 2. Variation in total stomatal area in normal (left) and flag (right) leaves of different wheat lines. Note boxes with same letter in each diagram are not significantly different (P<0.05) from one another.

The variation in total stomatal area under the microscopic field of vision in normal and flag leaves are presented in Fig 2. The total stomatal area was found to be greatest in Chinese Spring for both the normal and flag leaves. The total stomatal area in different disomic addition lines were mostly significantly lower (P<0.05) than in Chinese Spring.

Discussion

Stomata characteristics are not only governed genetically but by environmental factors too (Boyer *et al*. 1997; Woodward and Kelly 1995).

In case of cereals, flag leaf plays significant role in productivity (Biswal and Kohli 2013). In this study it was found that density and size were significantly different in normal leaves and flag leaves. In both cases stomatal density were found to be less on the lower surface. Providing the same experimental condition disomic addition line CS+5E'' showed comparatively greater density and minimal stomata size in both normal and flag leaf. Higher density in flag leaves may be due to the adaptive and developmental strategy of plants because in young condition the plants need less water for the transpiration so they develop few large stomata (Schletz, 2008). Large number of relatively small stomata in flag leaves may help conserve more water by minimizing water loss during grain filling period as this

process occurs during the near end of the growing period when the soil water content is minimal.

Miskin *et al.* (1972) reported variation in stomatal density and size on two surfaces of leaves of barley hybrids. Similar findings have also been reported in maize (Gaskell *et al.,* 1983). Finding of the present study are consistent with those of Miskin et al. (1972) and Gaskell *et al.* (1983).

Stomata plays a great role in controlling gas exchange and transpiration, but the process is regulated by various environmental factors such as light, temperature, CO_2 and water (Boyer *et al.,* 1997). The size and density of stomata are not the only factors for regulation water loss through transpiration. It has been found that the rate of transpiration is weakly correlated with stomata size (Maghsoudi and Maghsoudi, 2008). Similarly, stomatal density has a weak correlation with productivity, which in turn is affected by rate of transpiration, one of the outcomes of stomatal processes.

Addition lines contains the chromosome from *Th. elongatum* which is tolerant to several abiotic (Dvorak and Ross 1986) and biotic stresses (Shen and Ohm 2007; Sepsi *et al*., 2008). Wheat introgression lines containing the chromosome segment (7DL) from *Th. elongatum* have been reported to have improved water stress adaptations (Placido *et al*., 2013). The significant differences in various stomatal parameters between the control (Chinese Spring) and different disomic addition lines may also be possibly due to addition of stress tolerant genes present in those additional chromosomes. Study conducted by Quarrie and Jones (1977) in wheat leaves and Meng *et al.* (1999) in rice leaves suggest that stomatal size will decrease in drought. Stomatal length is also negatively correlated with density under different condition in leaves of *Platanus acerifolia* (Hao *et al.,* 2004).

Sutka *et al*. (1995) reported that CS+5E'' line was outstanding in drought tolerance in the experiment based on growth in different moisture regimes. Similar result were obtained by Rahmani *et al*., (2013) and Farshadfar *et al*., (2014) in a study based on QTLs controlling yield based indicators of drought tolerance in wheat-*Agropyron* disomic addition lines. The decrease in total stomatal area in most of the disomic addition lines compared to that in control (Chinese spring) in both the normal and flag leaves in present study also indicate towards their potential roles in water stress tolerance.

Conclusions

The stomatal development is a complex process controlled by a large number of genes. In *Arabidopsis* 40 different genes are known to contribute to stomatal development (Pillitteri and Torii, 2012). The differences in the degree of responses of different addition

lines in terms of various stomatal parameters under similar environmental conditions may be due to interaction of different genes for stomatal development in different lines. Introduction of chromosomes from alien species in wheat alters genetic composition along with the change in morphology and productivity. All the disomic addition lines showed some degree of changes in stomata characteristics compared to Chinese Spring. The changes in stomatal characteristics were significant in leaves of all lines except the flag leaves of CS+1E".

Acknowledgements

Conflict of interest

The authors declare no conflict of interest.

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