Leymus racemosus, a wheat wild relative is a potential source for wheat improvement for aluminum and heat stress tolerance

(コムギの近縁種、オオハマニンニクはコムギのアルミニウムおよび高温耐性を改良するための潜在的資源である)

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A thesis Submitted to the United Graduate School of Agricultural Sciences, Tottori University, in partial fulfillment of requirements for the doctoral degree (PhD) in Agricultural Sciences, Plant Molecular Breeding

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General introduction

Wheat (*Triticum* spp.) is one of the most widely grown food grain in the world, providing about one-fifth of the calories consumed by human (FAOstat 2007). Wheat is grown on more land area than any other commercial crop and continues to be the most important food grain source for humans. Its production leads all crops, including rice, maize and potatoes.

The world's population is expected to grow considerably over the coming years; the world's population is forecast to increase by 2 billion people to exceed 9 billion people by 2050. Recent FAO estimates indicate that to meet the projected demand, global agricultural production will have to increase by 60 percent from its 2005–2007 levels. In 2010–2012, about 870 million people or one in eight of the people in the world did not consume enough food to cover their minimum dietary energy requirements. Of these people, 852 million were in developing countries, making up 14.9 percent of the total population of these countries (FAOstat 2013).

This situation made the increase in wheat production area and wheat productivity by unit area an urgent objective worldwide. The total potential area for wheat production expansion estimated in the tropics to be between 3 and 4 million ha in nearly 60 countries. These countries can be divided into two basic environments; the first one is characterized by high temperature, dry, short crop season and few disease problems, whereas the second environment is characterized by high temperature, more humid, short crop season and high disease problems (Curtis 1988). The expansion of wheat production into these new marginal areas is hindered by biotic and abiotic stresses, therefore broadening the genetic

variability of wheat for more marginal areas and warmer climates and their stresses is essential. Moreover the expected reduction on wheat production due to climate change (Yang *et al*. 2013, Lobell *et al.* 2011) adding another dimension to the problem and complicate the future of food production.

Development of new stress tolerant cultivars with high yield potential and well adapted germplasm to enable the expansion and stabilization of wheat production and increase the wheat productivity, can be achieved by marker assisted breeding, genetic transformation and discovery and utilization of novel genes presented in wild relatives of wheat.

Several studies have revealed the significance of utilizing wheat alien genes to improve wheat abiotic stresses (heat, salt and drought), diseases, insect, kit-nematode resistance, nutrient use efficiency, grain yield and bread making quality (Eastwood *et al.* 1994, Jiang *et al.* 1994, Cox *et al.* 1995, Gatford *et al.* 2002, Marais *et al.* 1994, Martin-Sanchez *et al.* 2003, Dreccer *et al.* 2004, Wang *et al.* 2010, Garg *et al.* 2009, Li *et al.* 2013), with the aid of powerful molecular biology and cytogenetic techniques.

The nature of the most of the wild relatives as perennial species with huge biomass, small dormant seeds and poor agronomic traits complicate the proper evaluation and determination of their response under different stress conditions. On the other hand most of these wild relatives were successfully hybridized with wheat and resulted in wheat lines with addition, substitution and translocated chromosomes harboring part of the wild relative genome. The availability of hundreds of these lines in the genebanks facilitated the examination and determination of the wild relative's genes response under stress conditions

to explore new sources of stress tolerance genes. Moreover the presence of these genes in the wheat background allows the determination of their positive or negative impact when interacting with wheat genome.

One of the great sources of alien genes is the wheat wild relatives *Leymus* species. *Leymus* is a gnomically defined, allopolyploid, genus (2n=28 or 56; genome NsNsXmXm or NsNsNsNsXmXmXmXm) in tribe Triticeae, consisting of about 30 species worldwide. *Leymus racemosus* (2n= 28, NsNsXmXm) is a perennial grass that grows along sea coasts and in inland dry areas including saline or alkaline lands, dry or semi-dry areas and shady moist forests (Fan *et al*. 2009). It is evolutionarily distant from wheat, and has exceptionally large spikes, strong rhizomes and vigorous growth. *L. racemosus* is tolerant to salt and drought (McGuire and Dvorak 1981) and resistant to various diseases, such as scab (Mujeeb-Kazi *et al.* 1983). Our observations of *L. racemosus* plants growing in the Arid Land Research Center, Tottori, Japan (35°32′N, 134°13′E), also indicated its ability to tolerate heat stress; it showed vigorous growth, flowering from May to June, and filled its grains from July to August during the high temperature and humidity of the summer season. Several *Leymus* species including *L. racemosus* have been successfully hybridized and backcrossed with wheat. Some of the resulting addition lines possess potentially useful traits, including biological nitrification inhibition (Subbarao *et al.* 2007), resistance to *Fusarium* head blight (Chen *et al*. 2005, Qi *et al*. 2008, Wang and Chen 2008), and salt tolerance (Liu *et al*. 2001).

Due to the importance of *Leymus* as a novel source of useful traits, this thesis aimed to determine the impact of *L. racemosus* chromosomes on wheat aluminum toxicity

tolerance and heat stress tolerance and adaptation through physiological and molecular evaluation of wheat-*Leymus racemosus* chromosome introgression lines.

The first chapter describes the impact of several *L. racemosus* chromosomes on wheat aluminum tolerance and the identification of two introgression lines with enhanced aluminum tolerance. The second chapter demonstrates the effect of *L. racemosus* chromosomes on heat stress tolerance of wheat in term of physiological and morphological traits including yield and yield components. The third chapter shows the impact of two *L. racemosus* chromosomes on enhancing wheat adaptation by accelerating the flowering and maturity dates.

Chapter 1

Impact of wheat-*Leymus racemosus* added chromosomes on wheat aluminum tolerance

1.1 Abstract

Aluminum (Al) toxicity is the key factor limiting wheat production in acid soils. Soil liming has been used widely to increase the soil pH, but due to its high cost, breeding tolerant cultivars is more cost-effective mean to mitigate the problem. Tolerant cultivars could be developed by traditional breeding, genetic transformation or introgression of genes from wild relatives. Thirty wheat alien chromosome addition lines were used to identify new genetic resources to improve wheat tolerance to Al, and to identify the chromosomes harboring the tolerance genes. These lines and their wheat background Chinese Spring were evaluated for Al tolerance in hydroponic culture at various Al concentrations. Also Al uptake, oxidative stress, and cell membrane integrity were investigated. The *L. racemosus* chromosomes A and E significantly enhanced the Al tolerance of the wheat in term of relative root growth. At the highest Al concentration tested (200 µM), line E had the greatest tolerance. The introgressed chromosomes did not affect Al uptake of the tolerant lines. The improved tolerance conferred by chromosome E was attributing to improved cell membrane integrity. Chromosome engineering with these two lines could produce Al-tolerant wheat cultivars.

1.2 Introduction

Wheat (*Triticum aestivum*) is the major staple food crop in many parts of the world. Aluminum (Al) toxicity is the key factor limiting its production in acidic soils, which represent 40% of the world's cultivated land (Kochian 1995). Concentrations of soluble Al can reach up to 30 ppm in acidic soils with pH values below 5.5 (Evans and Kamprath 1970).

The exact mechanisms of Al toxicity are still not well understood. Al reduces root cell wall extensibility (Ma *et al.* 2004) and blocks Ca^{2+} channels of wheat root cell plasma membranes (Huang *et al.* 1992); it causes membrane damage and peroxidation of membrane lipids (Cakmak and Horst 1991, Wagatsuma *et al.* 1995). Al affects signal transduction pathways (Jones and Kochian 1997), blocks symplastic transport and communication in wheat roots by inducing callose deposition (Sivaguru *et al.* 2000), and causes mitochondrial dysfunction by triggering the production of reactive oxygen species in pea roots (Yamamoto *et al.* 2002).

Plants have developed strategies for detoxifying Al both externally and internally. Several mechanisms for external detoxification have been proposed (Kochian *et al.* 2004, Ma 2007, Poschenrieder *et al.* 2008). The most well studied is the secretion of organic acid anions, including citrate, oxalate, and malate, from the roots (Ma *et al.* 2001, Kochian *et al.* 2004). These anions chelate Al externally, preventing it from binding to root cells (Ma 2000). Genes encoding transporters for the Al-induced secretion of malate and citrate have been identified in many plants, including wheat malate transporter (*ALMT1*) (Ryan and Delhaize 2010). In some species such as buckwheat and hydrangea, internal detoxification

of Al is achieved by chelation with oxalate and citrate, respectively, and sequestration (Ma *et al.* 1997a, b, 2001). In Arabidopsis, the half-size ABC transporter ALS1 is implicated in Al sequestration (Larsen *et al.* 2007). *OsALS1* plays a crucial role in internal detoxification of Al and tolerance in rice (Huang *et al*. 2012).

Soil liming is used widely to raise soil pH and prevent Al toxicity, but its high cost and other effects on soil properties make the use of tolerant cultivars a more cost-effective and environmentally friendly solution. Significant improvements in the Al tolerance of wheat have been achieved by conventional breeding methods, but the genetic variation of this tolerance in wheat is limited. Within the wild members of the tribe Triticeae, higher levels of tolerance have been identified in the *Aegilops uniaristata* ($2n = 2x = 14$, NN) (Berzonsky and Kimber 1986) and introduced successfully into wheat (Miller *et al*. 1997).

Due to the importance of *Leymus* species generally and *L. racemosus* particularly as novel sources for many economically important traits this study was intended to investigate the effect of *Leymus*-derived chromosomes (thereafter designated as *Leymus* added chromosomes) on wheat Al tolerance. This study describes the effect of *L. racemosus* added chromosomes on wheat Al tolerance and the identification of two lines with enhanced Al tolerance.

1.3 Materials and methods

1.3.1 Plant materials and growing conditions

I studied 13 wheat-*L. racemosus* addition and 2 wheat-*L. racemosus* substitution lines (Table 1-1), in addition to 15 addition lines harbor the homoeologous group 2 (HG 2) chromosomes from 11 species: *L. mollis* (2 lines), *Aegilops longissima* (2 lines), *Ae.*

geniculata (2 lines), *Ae. peregrina* (2 lines), *Ae. umbellulata* (1 line), *Ae. searsii* (1 line), *Agropyron elongatum* (1 line), *Hordeum chilense* (1 line), *Secale cereale* (1 line), *Elymus ciliaris* (1 line) and *Psathyrostachys huashanica* (1 line). These lines and their wheat recipient cultivar 'Chinese Spring' (CS) were provided by the Tottori Alien Chromosome Bank of Wheat (TACBOW) supported by National BioResource Project–Wheat.

The seeds of all lines were surface-sterilized in sodium hypochlorite solution (1.2% v/v) for 10 min. After several washes with deionized water, the seeds were soaked in distilled water for 12 h and then transferred to Petri dishes to germinate in the dark for 24 h. The germinated seeds were transferred to a mesh floating on aerated 200 μM $CaCl_2$ solution (pH 4.6) in 20-L plastic containers. All experiments were carried out in a glasshouse at the Arid Land Research Center (Tottori, Japan; 35°32′N, 134°13′E) at a constant 22 °C under natural light during winter. Three-day-old seedlings of uniform length were used to determine the best Al concentration for the evaluation of the addition lines, whereas four-day-old seedlings were used for other experiments. In all treatments, a solution of 200 μM $CaCl_2$ was used as a background electrolyte. During the experiment, the solutions were adjusted to pH 4.6 and renewed daily.

1.3.2 Evaluation of Al tolerance

The primary lengths of the longest root of each 4-day-old seedling was measured, then seedlings were placed in 0 (control) or 25 μM $AlCl_3 \cdot 6H_2O$ culture solution. After 48 h, the longest root on each plant was measured again, and the net root growth per plant was calculated. Root growth was expressed as relative root growth (RRG) = 100 (RG_{al}/RG_c),

where RG_{al} represent the net root growth with Al treatment and RG_c represent net root growth without Al.

In the dose response experiment, 4-day-old seedlings were exposed to 0, 50, 100, or 200 µM $AlCl_3 \cdot 6H_2O$ for 48 h, and then the roots were measured as above for calculation of RRG. In the prolonged effect experiment, seedlings were exposed to 10 µM $AlCl_3 \cdot 6H_2O$ for 5 days. Roots were measured and RRG was determined for every 24-h period as above.

1.3.3 Aluminum distribution in root tissues

Localization of Al in root tips was determined by staining with Morin (Sigma-Aldrich, St. Louis, MO, USA), which is used widely to detect the presence and distribution of Al in root tissues (Tice *et al.* 1992). After exposure to 0 or 25 µM $AlCl_3 \cdot 6H_2O$ for 48 h, root tips (1 cm) were excised, washed for 10 min in 5 mM NH_4OAc buffer (pH 5), stained in 100 µM Morin in 5 mM NH_4OAc buffer (pH 5) for 1 h, and washed again in NH_4OAc buffer for 10 min. The stained root tips were examined under an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a BP 400–440-nm excitation filter and an LP 470-nm barrier filter. Fifteen root tips from five seedlings in each treatment were examined, and the experiment was repeated three times.

1.3.4 Determination of aluminum contents in roots

Al content in root tips was determined according to Osawa and Matsumoto (2001). Excised 1-cm root tips (20 mg) were placed in a microcentrifuge tube (1.5 mL) containing 1 mL of 2 M HCl. The tubes were placed on an orbital shaker at 10 rpm for 24 h to release the Al from the root apices. After dilution, the Al content in the HCl solution was determined by atomic absorption spectrophotometry (AA-6800, Shimadzu, Kyoto, Japan).

1.3.5 RT- PCR analysis

Total RNA was extracted from the roots of Al treated and non-treated seedlings after 48 h of 25μM Al treatment using TriPure isolation reagent (Roche, Mannheim, Germany), following the manufacturer instructions. RNA was treated with RNase-free DNase 1 (Takara, Ohtsu, Japan) to remove any genomic DNA. 1 μg RNA was used to synthesize first strand cDNA using Transcriptor first strand cDNA synthesis Kit (Roche). The first strand cDNA (50 ng) was used for the PCR using primers 5'-CGTGAAAGCAGCGGAAA-GCC-3' and 5'-CCCTCGACTCACGGTACTAACAACG-3' for amplification of the *ALMT1* transcript (Raman *et al*. 2005), and primers 5'-TCAACGAGGAATGCCTAG-TAAGC-3' and 5'-ACAAAGGGCAGGGACGTAGTC-3' for the amplification of the ribosomal 18S gene as internal control gene (Fontecha *et al.* 2007). The PCR conditions were initial denaturation at 95 °C for 5 min followed by 35 cycles at 94 °C, 58 °C and 72 °C for 30 seconds each then final extension step at 72 °C for 7 minutes. Additionally, we examined the expression patterns of some genes associated with wheat Al tolerance including citrate transporter, ent-kaurenoic acid oxidase (KAO1), P450 monooxygenase CYP72A26, beta-glucosidase aggregating factor, lipid transfer protein-like protein 1 (Table 1-2).

1.3.6 Evaluation of the tolerance to long-term Al toxicity

Based on the above experiments, 2 tolerant addition lines and CS were selected for further evaluation. Seeds were sterilized, soaked and germinated as described above. One-week-old uniform seedlings were transplanted to 20 L containers containing 15 L of 1/8 strength Hoagland solution (pH 4.6) in which the phosphate strength was 1/16. On the 4th

day after transplanting, two treatments were established: Control (1/8 Hoagland solution, pH 4.6), and Al solution (1/8 strength Hoagland solution containing 300 µM Al, pH 4.6). The solution was changed daily, and its pH was adjusted to 4.6. After 15 days of treatment, numbers of tillers per plant (TP), chlorophyll content (ChC), root dry weight (RDW), shoot dry weight (SDW), root Al content and shoot Al content were measured. Chlorophyll content was estimated on the upper most expanded leaves using a chlorophyll meter SPAD-502 (Konica Minolta, Japan). Plants were harvested, dried at 60ºC for 3 days then SDW and RWD were measured. For evaluation of Al tolerance the resistance integrated score formula used by Dai *et al.* (2011) to evaluate the Al resistance of wild barley germplasm exposed to 100 µM Al for 15 days was adopted: Resistance integrated score = absolute value of (SPAD value x 0.2 + tillers/ plant x 0.2 + shoot dry weight x 0.2 + root dry weight x 0.2).

Al was extracted from 100 mg dry root or shoot tissues from the Al treated plants as described by (Yin *et al.* 2010). The Al concentration was measured by an inductively coupled plasma atomic emission spectrometer (ICP-AES, Ciros CCD, Rigaku, Japan).

1.3.7 Visualization of lipid peroxidation

Aldehydes, products of lipid peroxidation were detected histochemically by Schiff's reagent (Yamamoto *et al.* 2001). Root tips exposed to 0 or 25 µM $AlCl_3 \cdot 6H_2O$ for 48 h were excised and stained immediately in Schiff's reagent (Wako, Osaka, Japan) for 20 min and then rinsed with a freshly prepared sulfite solution (0.5% w/v $K_2S_2O_5$ in 0.05 M HCl). The root tips were kept in the sulfite solution until observation under a light stereomicroscope (Olympus SZX16).

1.3.8 Plasma membrane integrity assay

Electrolyte leakage was used as an indicator of the loss of plasma membrane integrity (Singh *et al*. 2007). Root tips (20 mm) exposed to 0 or 25 µM Al for 24 or 48 h were incubated in distilled water at 25°C for 2 h in tubes, and then the electrical conductivity (EC1) of the medium was measured using Horiba B-173 conductivity meter (Horiba, Kyoto, Japan). The tubes containing the root material were then boiled for 30 min to release all the electrolytes, and cooled at room temperature to 25°C before the final electrical conductivity (EC2) measurement. Electrolyte leakage was calculated as 100 × [1-(EC1/EC2)].

1.3.9 Visualization of plasma membrane integrity

Root tips exposed to 0 or 25 µM Al for 48 h were excised and stained immediately in aqueous Evans blue (Sigma-Aldrich) solution (0.025% w/v) for 10 min (Yamamoto *et al*. 2001). Stained roots were washed three times with distilled water, after which the dye no longer eluted from the roots. Intact stained roots were observed under a light stereomicroscope (Olympus SZX16). Fifteen roots from five seedlings in each treatment were examined, and the experiment was repeated three times.

1.3.10 H_2O_2 detection and determination

The distribution of H_2O_2 in the root tips was detected by the florescent dye 2,7-dichlorofluorescein diacetate, DCF-DA (Wako Pure Chemical, Osaka, Japan) as described by (Jones *et al.* 2006). Root tips exposed to 25 µM Al for 48 h were excised and placed into a solution containing 200 mM $CaCl_2$ (pH 4.6) and 10 mM DCF-DA for 15 min, then DCF-DA fluorescence was detected under an Olympus BX51 microscope (excitation 488 nm, emission 530 nm).

1.3.11 Statistical analyses

In each experiment, fifteen replicated seedlings were used for each line and each experiment was conducted twice. All values are shown as means ± the standard error of the mean (SEM). Data were analyzed by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test at $P < 0.05$. Statistical analysis was performed with StatView software v. 5.0.1 (SAS Institute, Inc., USA).

1.4 Results

1.4.1 Effect of aluminum on root growth

To identify the best concentration to screen the addition lines, I examined the Al tolerance of the moderately Al-tolerant CS (Aniol 1990) at 0, 25, 50, 100, 200, and 400 µM Al for 48 h. Relative root growth (RRG) decreased with increasing Al concentration. To screen the addition lines, we selected 25 µM, which reduced the RRG of CS by about 50% (Fig. 1-1A).

In the screening of wheat-*Leymus racemosus* addition lines at 25 µM Al for 48 h, addition lines A, E and O showed better tolerance to Al in term of significantly (P<0.05) higher RRG compared to CS (Fig. 1-1B). Addition lines A, E and O had 94, 77 and 79% RRG, respectively, compared to only 57% in CS (Fig. 1B, C). On the other hand, the RRG of lines H, N, R, and S was significantly lower than that of CS (Fig. 1-1B).

The three addition lines (A, E and O) showing the highest RRG, one addition line (Hs) comparable to CS and one line (H) exhibiting low RRG were selected and evaluated in dose response experiment to confirm the tolerance of A, E and O, and to determine to which level those lines can tolerate Al toxicity. After exposure to 50, 100, or 200 µM Al for

48 h, RRG was highest in lines A, E and O, and lowest in lines H, N and Hs (Fig. 1-2). At 50 µM Al, RRG was significantly higher in A, E, and O than in CS. At 100 and 200 µM Al, only in E it was significantly higher than in CS; at 200 µM Al, RRG was 17% higher in E than in CS.

Lines A and O are phenotypically similar in heading and maturity time, root characteristics, and seed shape (data not shown). As these two lines harbor the same HG 2 chromosome of *L. racemosus* (Larson *et al.* 2012), I selected A and E for further characterization.

To examine the effect of low Al concentration for longer treatment time, CS, A and E were evaluated under 10 µM Al for 5 days. After 1 day in 10 µM Al, RRG was enhanced in all 3 genotypes, with greater enhancement observed on lines A and E (Fig. 1-3). On day 2, RRG had declined slightly in CS, whereas line E maintained its growth enhancement, and line A maintained a small advantage. On day 3, RRG had declined by 24% in CS, while no reduction was apparent in lines A and E. On days 4 and 5, although their root growth was reduced relative to the control, RRG in lines A and E remained 20% and 15% higher, respectively, than that in CS. Throughout the 5-day experiment, the addition lines maintained significantly ($P<0.05$) higher RRG compared to CS, except on day 2, when line A was comparable to CS.

To know the effect of other alien chromosomes belonging to HG 2 on Al tolerance, the 15 HG 2 chromosome addition lines were tested at 25µM Al. The result indicated that all the tested lines had RRG comparable to CS, and none of the added chromosomes form the 11 species enhanced or reduced the tolerance of CS (Fig. 1-4).

1.4.2 Al content and localization in the root tips, and ALMT1 expression

To investigate whether the enhancement of Al tolerance in the addition lines was associated with an increased ability to exclude Al from the root tips, the Al contents were determined in the root tips of CS, A, E, F, Hs and P. Significantly ($P<0.05$) higher Al contents were detected in root tips of lines F, Hs and P (Fig. 1-5A). In contrast, addition lines A and E accumulated similar amounts of Al as CS. Staining with the highly Al-sensitive fluorescent dye Morin (Tice *et al.* 1992) confirmed that CS and lines A and E accumulated the similar amounts of Al (Fig. 1-5B).

As there is no sequence information available for *L. racemosus* to enable the detection of the expression of Al tolerance genes, the expression of some wheat Al tolerance-related genes was examined to investigate the effect of the added chromosomes on the expression of those genes. While no difference on the expression of *ALMT1* was detected in CS, a reduced expression upon Al treatment was observed on addition lines A and E (Fig. 1-5C). In contrast, lines F, Hs and P showed the same level of expression of CS (Fig. 1-5D). The expression of other Al tolerance related genes were examined in CS, A, and E, and the results indicated that the three lines had comparable expression levels.

1.4.3 Long term effect of aluminum

To test the suitability of using these lines for breeding, CS and addition lines A and E were selected and the effects of Al on different plant growth parameters evaluated. Although treatment with 300 µM Al for 15 days resulted in a reduced chlorophyll contents in all tested lines, addition lines A and E suffered significantly ($P<0.05$) less percentage reduction on chlorophyll contents compared to CS (Table 1-3). Line E had lower reduction

in the number of tillers per plant compared to line A and CS. No significant differences were observed in SDW and RDW between both addition lines and CS. The resistance integrated score was calculated as an indication of Al tolerance. The two addition lines had significantly higher integrated score than CS (Table 1-3). No significant differences were observed in Al contents in the roots of the addition lines and CS, whereas, line E accumulated significantly higher Al content in the shoots compared to CS and line A. Line E accumulated 1.36 and 1.5 fold Al than line A and CS, respectively.

1.4.4 Assessment of cell membrane integrity, cell viability, lipid peroxidation, and H_2O_2 accumulation and distribution

I examined the cell membrane integrity of CS, lines A, and E by detecting the amount of ion leakage. Treatment with 25 μM Al for 24 h reduced the cell membrane integrity of CS significantly, but not that of lines A and E (Fig. 1-6A). Treatment for 48 h reduced the integrity in the three lines significantly, by 35% to 47% (Fig. 1-6B). Addition line E had the lowest reduction in cell membrane integrity whereas A had the highest reduction in cell membrane integrity compared to CS. The result of the 48 h treatment is consistent with staining with Evans blue, which detects the magnitude of cell death: The root tips of the seedlings grown without Al did not absorb the dye, indicating no damage in the root cells (Fig 1-7A). On the other hand, the root tips of the seedlings treated with 25 μM Al for 48 h were affected by Al. The magnitude of the damage was similar in all tested lines.

Lipid peroxidation was evaluated using Schiff reagent. No Schiff staining was detected in plants grown without Al. After 48 h of Al treatment, no clear differences in the accumulation of aldehydes were observed in the elongation zone of lines A, E and CS (Fig.

1-7B).

DCF-DA staining indicates that line A slightly accumulated more H_2O_2 than CS and line E when grown without Al (Fig 1-7C). In the presence of Al, the amount of H_2O_2 was increased in all lines with no clear differences in H_2O_2 accumulation between the three lines. These results reveal no differences between the addition lines and CS in lipid peroxidation and oxidative stress. Line E had the highest cell membrane integrity, and CS and line A were comparable to each other.

1.5 Discussion

1.5.1 Effect of L. racemosus chromosomes on root elongation under Al stress

Reduction of root elongation is the first visible symptom of Al toxicity and can be used to examine Al sensitivity among genotypes (Sasaki *et al*. 1994). Chromosome addition lines A and E showed the best Al tolerance in term of higher RRG in these lines compared to CS under all tested Al concentrations. At 25 and 50 µM Al, line A performed better than line E (Figs. 1-1B and 1-2), while at higher concentrations tested, E performed better (Fig. 1-2). Kinraide (1993) concluded that low concentrations of Al often enhance root growth in wheat, and the magnitude of the enhancement is correlated with the level of Al tolerance. At the lowest Al concentration (10 µM) tested in this study, RRG in the two addition lines was enhanced on days 1 and 2 of the treatment, and in CS on day 1 only; RRG in CS started to decline from day 2 (Fig. 1-3). Therefore, this result could be considered as an evidence for the tolerance of lines A and E.

Both lines A and O seemed to harbor the same homologous chromosome of *L. racemosus* (Larson *et al.* 2012). However, line A was developed by Kishii *et al*. (2004) in

Japan, whereas line O was developed by Qi *et al*. (1997) using different *L. racemosus* strain in China. The tolerance of line A at 25 µM Al was better than that of line O, despite the similarity of their phenotype, root characteristics, seed shape, and days to heading and maturity. This concludes that the difference of Al tolerance is attributable to the allelic differences in the *L. racemosus* strains used to develop the addition lines. Line T is a substitution line including the same *L. racemosus* chromosome as A and O in place of wheat chromosome 2B (Qi *et al.* 1997). Firstly it was assumed that it would show similar tolerance as lines A and O, however, it exhibited the same level of tolerance as CS, perhaps owing to the absence of chromosome 2B. Gustafson and Ross (1990) studied the effect of wheat chromosomes arms on the expression of Al tolerance using hybrids between Al tolerant rye and ditelocentric lines of CS. They concluded that the tolerance of rye when expressed in wheat was evidently under the influence of genes located on a number of wheat chromosomes, and that the absence of some chromosome arms allowed the expression of tolerance, and the absence of other chromosome arms hindered it. Accordingly, the result suggests the importance of wheat chromosome 2B in the expression of the Al tolerance of *L. racemosus* chromosome A.

The RRG was significantly lower in lines N, R, H and S than in CS (Fig. 1-1B), indicating that their introgressed chromosomes reduced the tolerance to Al in a wheat background and these chromosomes has inferior effect. The rest of the addition lines had the same level of tolerance as CS indicating that the added chromosomes do not have an effect on the Al tolerance.

The Al-tolerance-related genes in CS are located on chromosome arms 6AL, 7AS,

2DL, 3DL, 4DL, and 4BL and on chromosome 7D (Aniol and Gustafson 1984, Papernik *et al*. 2001). Considering the synteny between *L. racemosus* and wheat chromosomes (Qi *et al*. 1997, Kishii *et al*. 2004), I expected the presence of some tolerance genes in lines A (HG 2), F (HG 4), H (HG 3), K (HG 6), N (HGs 3, 7), O (HG 2), and T (HG 2). Kishii *et al.* (2004) could not assign chromosome E to any group, as only one marker present on HG 4 was available. These results indicate that only lines A and O both belonging to HG 2 were tolerant. Fifteen addition lines from 11 species all of which harbor HG 2 chromosomes were screened for Al tolerance and none of them possess tolerance to Al. This result indicates that the tolerance of lines A and O is not due to genetical imbalance by presence of extra HG 2 chromosomes but due to specific gene(s) on *L. racemosus* chromosomes. Additionally, the addition line of *L. mollis*, a related species of *L. racemosus*, did not show any tolerance despite their similar morphology. This finding also indicates that the Al tolerance of lines A and O is due to specific gene(s) on the *L. racemosus* chromosomes.

1.5.2 Effect of L. racemosus chromosomes on Al accumulation

Quantification of Al after 48-h and after 15-days Al treatments indicated that lines A and E accumulated the same amount of Al as CS (Fig. 1-5A, B; Table 1-3). So the mechanism behind the tolerance of lines A and E must not rely on enhanced ability of Al exclusion from the root tips. The expression of the *ALMT1* was not induced by Al treatment in CS and was down regulated in the addition lines A and E. Using RT-PCR analysis Fontech *et al.* (2007) reported that the expression of *ALMT1* is not induced by Al in CS. Sasaki *et al.* (2004) and Raman *et al.* (2005) also reported that tolerant wheat genotypes Atlass 66, ET8 and CS express *ALMT1* constitutively and are not affected by Al. Ryan *et*

al. (1995) mentioned that the Al tolerance in wheat is strongly correlated with the capacity for Al-activated malate efflux. These results and the results of Al accumulation suggest presence of another mechanism operating in the addition lines and maintaining the same amount of Al in the roots. Ryan *et al* (2009) indicated that the tolerance of wheat cultivar Carazinho relied on constitutive efflux of citric acid. Yang *et al*. (2011) reported that the rhizosphere pH regulation by plasma membrane H^+- ATPase was associated with the relative root elongation and Al content in root apex of tolerant cultivar ET8. In rice the cell wall polysaccharides were responsible for Al exclusion from the root tips of cultivar Nipponbare (Yang *et al.* 2008). In buckwheat higher levels of Al-phosphate complexes might be presented in the apoplast of the Al-tolerant cultivar, suggesting a novel mechanism of Al exclusion from the cytoplasm (Zheng *et al.* 2005).

After 15 days of Al treatment, line E translocates the highest amount of Al to the shoots than CS and A (Table 1-3). The amount of Al translocated to the shoots is 102 mg kg^{-1} DW. According to Foy (1984), Al accumulator plants have been defined as they accumulate more than 1000 mg kg^{-1} Al in leaves. Therefore we conclude that the enhanced tolerance of line E is not associated with Al accumulation in the shoot parts.

Several studies have reported a positive correlation between *ALMT1* expression and Al tolerance. Enhanced *ALMT1* expression results in reduced Al accumulation (Sasaki *et al.* 2004, Raman *et al.* 2005). Lines F, Hs and P accumulated more Al than CS (Fig. 1-5A, B), but they exhibited similar *ALMT1* expression (Fig. 1-5D) and comparable Al tolerance to that found in CS (Fig. 1-1B). These results suggest the presence of other Al tolerance mechanism in these addition lines.. In buckwheat and hydrangea, Al is chelated internally

by oxalate and citrate, respectively (Ma *et al.* 1997a, b, 2001). A half-size ABC transporter ALS1 is implicated in Al sequestration in Arabidopsis (Larsen *et al.* 2007) and rice (Huang *et al*. 2012). Regulation of hormonal equilibrium in plants by nitric oxide has been suggested to enhance Al tolerance (He *et al*. 2012).

No differences were detected between CS and lines A and E in cell death, lipid peroxidation and H_2O_2 distribution and accumulation. These results indicate that the tolerance of addition lines A and E is not due to enhanced capacity to mitigate the oxidative stress caused by the Al treatment.

In conclusion the addition of *L. racemosus* chromosomes A, O and E to wheat enhanced the tolerance to Al, whereas the addition of chromosomes N, R, and S reduced the tolerance, and the addition of the rest of the chromosomes did not affect the tolerance. In the case of chromosome A, the mechanism remained to be clarified in details in future studies. In the case of chromosome E, it might be increased cell membrane integrity (Fig. 1-6). Wide hybridization and chromosome engineering with these two addition lines could produce more Al-tolerant wheat cultivars. This work should be continued to clarify the mechanism behind the Al tolerance in lines A and E. Deletion mapping should be carried out and translocated lines should be produced to facilitate the transfer of chromosome parts that confer Al tolerance to wheat.

Table 1-1. List of the wheat-*Leymus racemosus* chromosome addition lines and their chromosomes names and homoeologous groups.

Strain ID	Strain name and chromosome name	Homoeologous group	Number of chromosomes	Designation in this experiment	Reference
TACBOW0001 [a]	*Leymus racemosus* A addition	2	44	A	Kishii *et al.* 2004
TACBOW0003	*L. racemosus* E addition	ND [b]	44	E	Kishii *et al.* 2004
TACBOW0004	*L. racemosus* F addition	4	44	F	Kishii *et al.* 2004
TACBOW0005	*L. racemosus* H addition	3	44	H	Kishii *et al.* 2004
TACBOW0006	*L. racemosus* I addition	5	44	I	Kishii *et al.* 2004
TACBOW0008	*L. racemosus* k addition	6	44	K	Kishii *et al.* 2004
TACBOW0009	*L. racemosus* l addition	2	44	L	Kishii *et al.* 2004
TACBOW0010	*L. racemosus* n addition	3,7	44	N	Kishii *et al.* 2004
TACBOW0011	*L. racemosus* H substitution	3	42	Hs	Kishii *et al.* 2004
TACBOW0012	*L. racemosus* 2Lr#1 addition	2	44	O	Qi *et al*. 1997
TACBOW0013	*L. racemosus* 5Lr#1 addition	5	44	P	Qi *et al*. 1997
TACBOW0014	*L. racemosus* 7Lr#1 addition	7	44	Q	Qi *et al*. 1997
TACBOW0015	*L. racemosus* 7Lr#1 addition	7	44	R	Qi *et al*. 1997
TACBOW0016	*L. racemosus* ?Lr#1 addition	ND	44	S	Qi *et al*. 1997
TACBOW0017	*L. racemosus* 2Lr#1 substitution	2	42	T	Qi *et al*. 1997

[a]TACBOW: Tottori Alien Chromosome Bank of Wheat supported by NBRP-wheat; [b]ND, not determined.

Table 1-2. Oligonucleotide sequences used for RT-PCR in this study

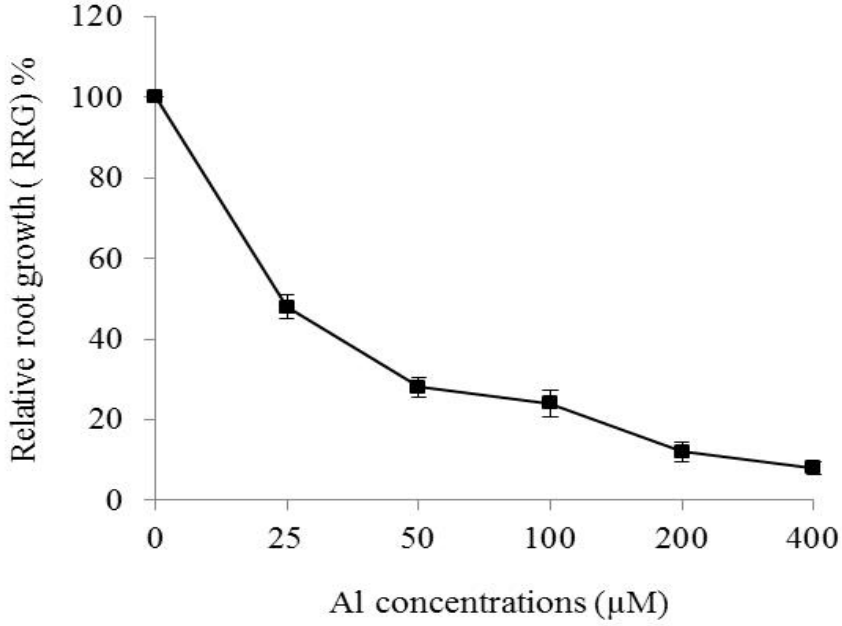
Gene	Sequence		Reference
	Forward	Reverse	
Citrate transporter	GATTGCCGCGACCTCTCGTGTT	GATGCCGTCGAACACGAACG	Ryan *et al.* 2009
Cytochrome P450-like protein	CAAGCTCGTCATTCTCCACCTC	CGAAGTTGACCAAGATGGAATACTG	Guo *et al.* 2007
Ent-kaurenoic acid oxidase, KAO1	GGCTACACCATACCGAAGGGA	CTATCAGGATTGAAGGAGAGAGGATC	Guo *et al.* 2007
P450 monooxygenase CYP72A26	GTATGTGGCGCATGAGTATACGAC	GGTCAGAATTTTGCACTGCTTG	Guo *et al.* 2007
Beta-glucosidase aggregating factor	AATAACTGGGCCATAGTTGATGC	TAATAGCCAACACTTGGTTGATCAG	Guo *et al.* 2007
Lipid transfer protein-like protein 1	CTGTTACAGTTCGTATGTCAGGCG	CCATAACAGGATACAATGACATTGATC	Guo *et al.* 2007

Table 1-3. Percent reduction from control in various growth parameters of CS and addition lines A and E grown for 15 days at 300 μM Al.

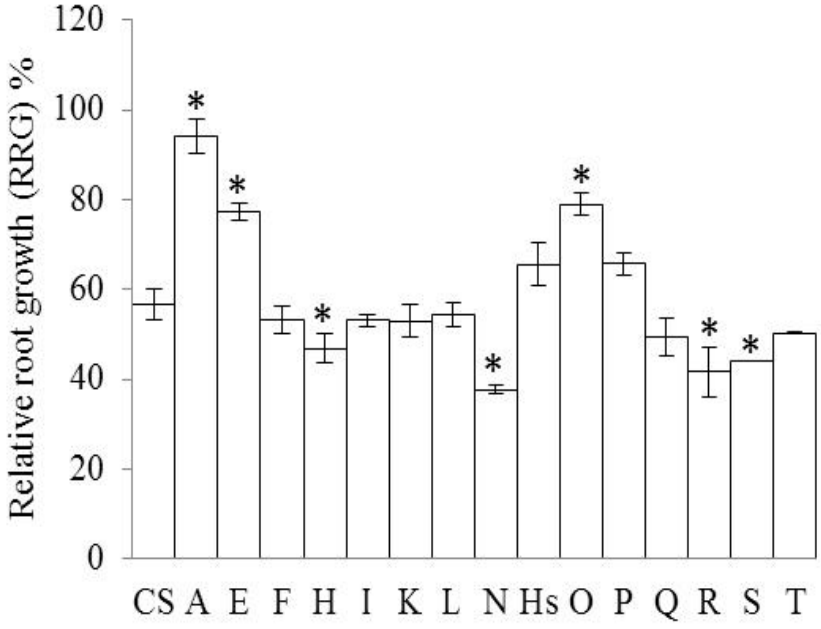
	% Reduction from control					Al contents (mg kg^{-1} DW)	
	ChC [a]	TP	SDW	RDW	RIS	Root	Shoot
CS	35 ± 3.2	33 ± 4.2	34 ± 2.8	-0.05	5.8 ± 0.1	4911 ± 172.6	67 ± 4.4
A	20 ± 1.0*	32 ± 0.2	37 ± 3.4	0.02	6.7 ± 0.1*	4661 ± 122.5	75 ± 5.9
E	21 ± 1.9*	27 ± 7.8	39 ± 6.4	-0.03	6.6 ± 0.05*	5159 ± 126.9	102 ± 10

[a] ChC, chlorophyll content; TP, tiller number per plant; SDW, shoot dry weight; RDW, root dry weight; RIS, resistance integrated score. Values are presented as means ± SEM (n=2); each replicate included 7 replicated seedlings. Asterisks indicate the significant difference from CS ($P < 0.05$).

A


Relative root growth (RRG) %
120
100
80
60
40
20
0
0
25
50
100
200
400
Al concentrations (µM)

B


Relative root growth (RRG) %
120
100
80
60
40
20
0
CS A E F H I K L N Hs O P Q R S T

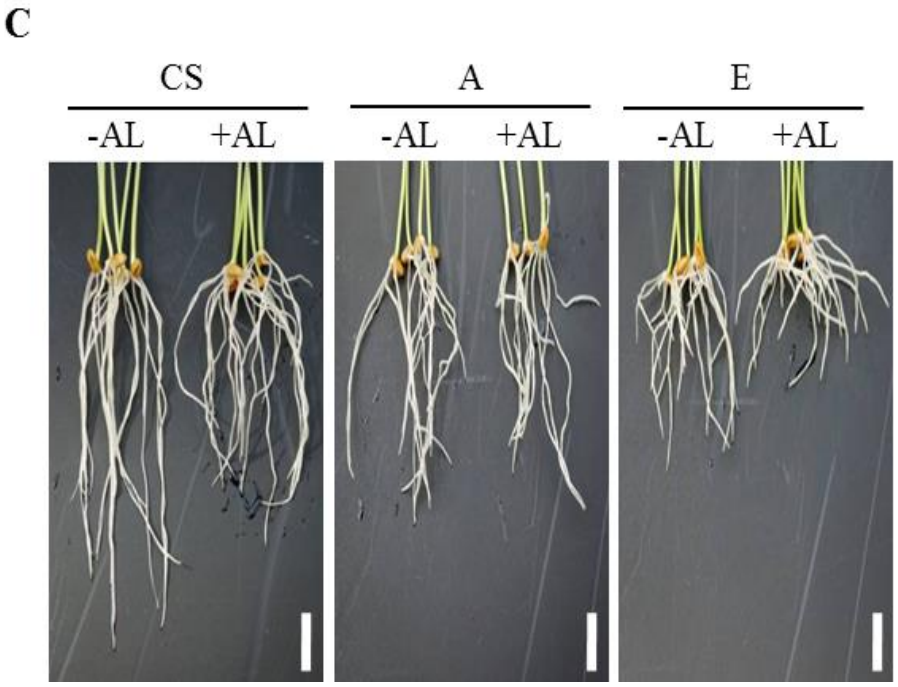


Fig. 1-1. Effect of Al on root growth of CS and *Leymus racemosus* chromosome addition lines. (A) The effect of different concentration of Al in relative root growth (RRG) of CS. Seedlings were exposed to 0, 25, 50, 100, 200, 400 μM Al for 48 h. Values are the mean of 10 replicated seedlings and the vertical bars represent the SEM. (B) Relative root growth (RRG) of CS and addition lines grown in 25 μM Al for 48 h. Values are means ± SEM (*n* = 2) of growth with Al over growth without Al; each replicate included 15 seedlings. Asterisks indicate significant differences from CS ($P<0.05$, Fisher's PLSD test). (C) Sensitivity of CS and lines A and E to 25 μM Al for 48 h. Scale bar = 5 mm.

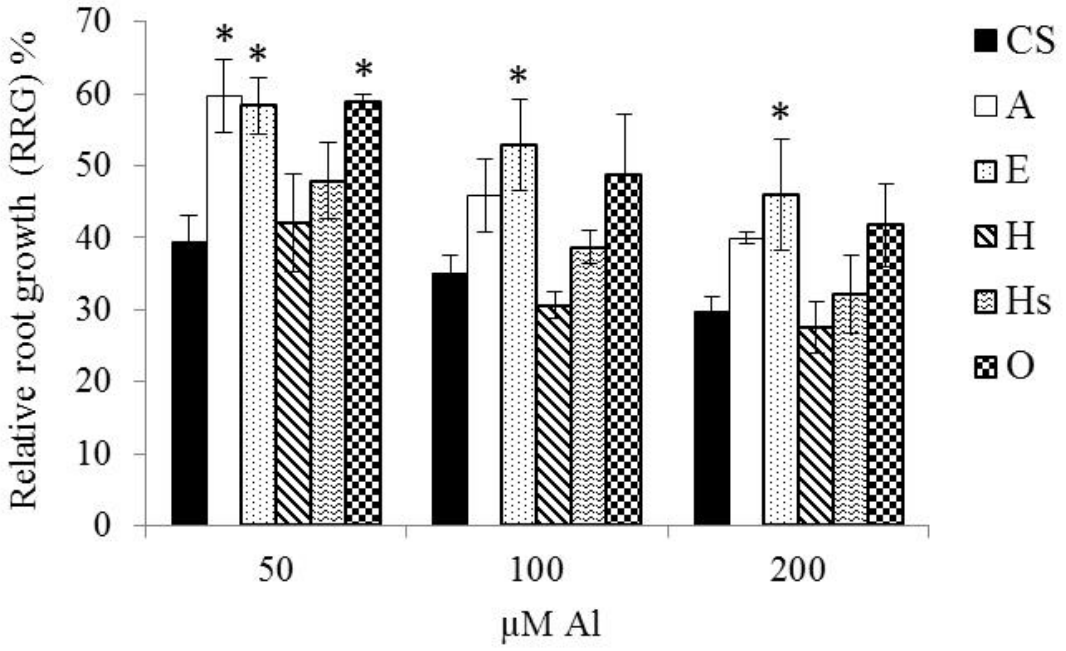


Fig. 1-2. Dose response evaluation of relative root growth (RRG) of CS and selected addition lines. Seedlings were exposed to 50, 100, or 200 µM Al for 48 h. Values are means ± SEM (n = 2); each replicate included 12 seedlings. Asterisks indicate significant differences from CS (P<0.05, Fisher's PLSD test).

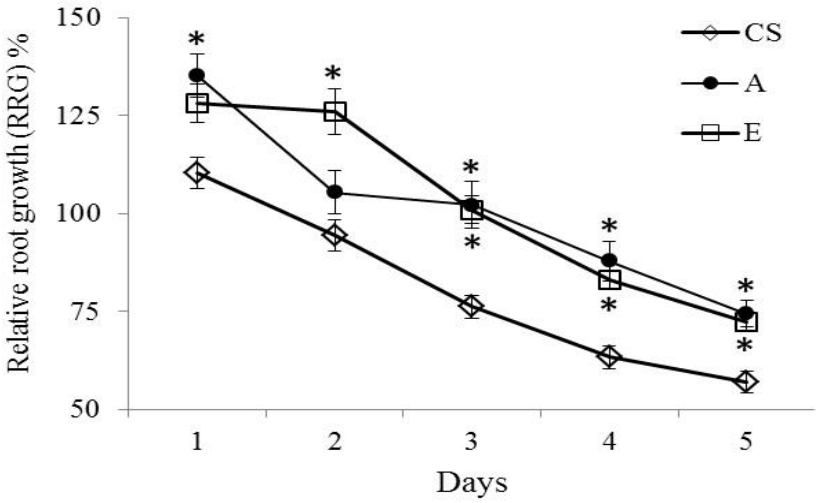


Fig. 1-3. Time course of effects of Al on CS and addition lines A and E. Seedlings were exposed to 10 μM Al for 5 days and the relative root growths were measured daily. Values are means ± SEM (n = 15) of the relative root growth (RRG) for each 24-h period. P<0.05, Fisher's PLSD test.

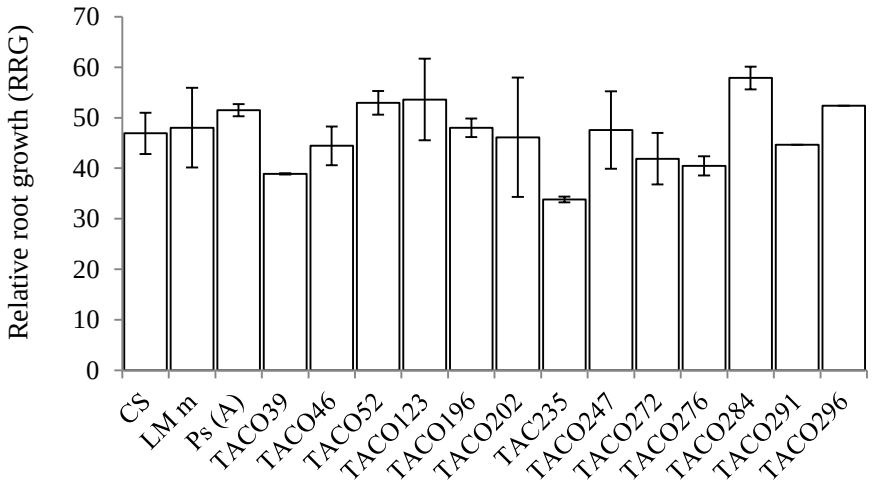


Fig. 1-4. Effects of Al on CS and HG2 addition lines exposed to 25 μM Al for 48 h. Values are means ± SEM (n = 2) each replicate included 10 seedlings. P<0.05, Fisher's PLSD test.

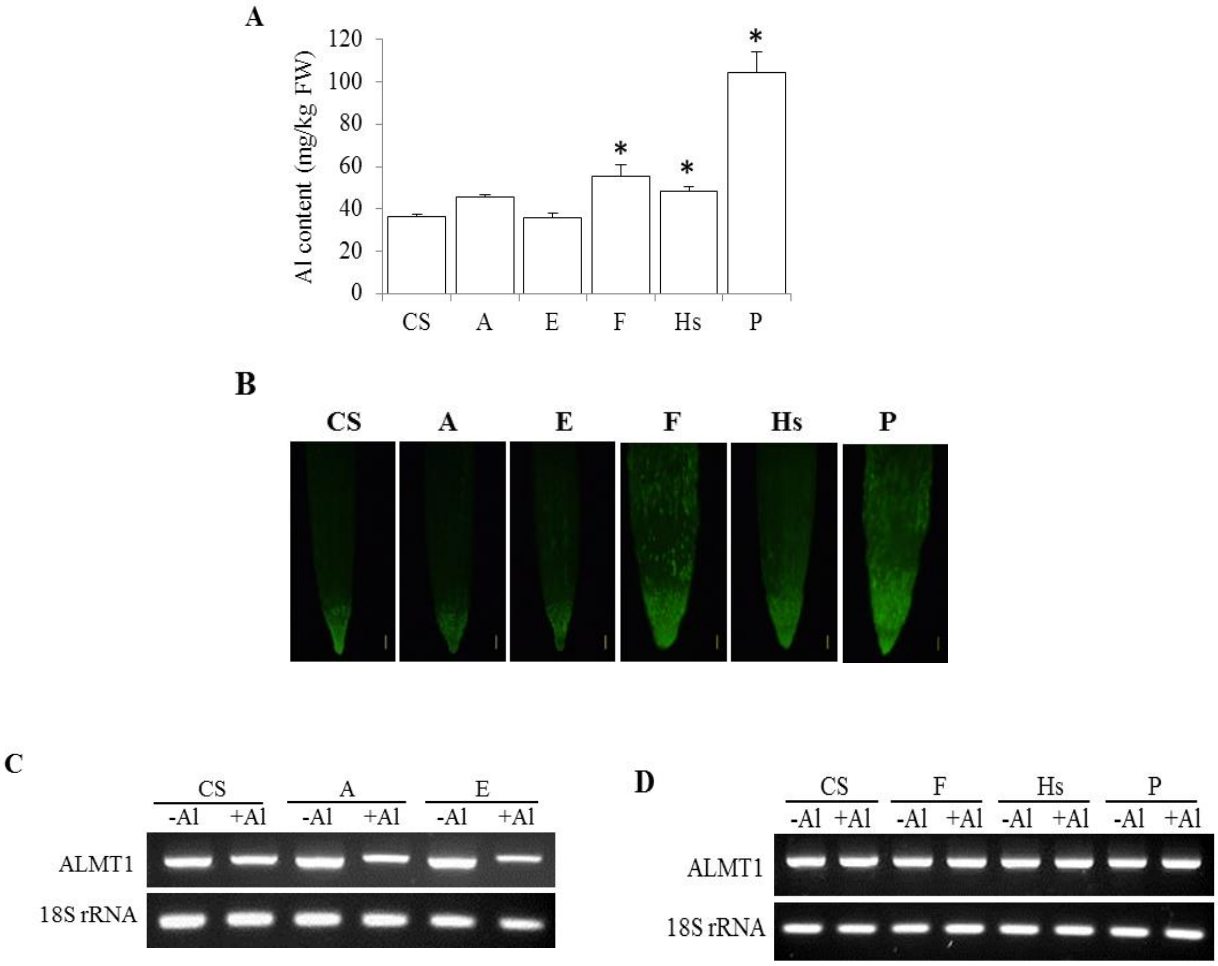


Fig. 1-5. Al accumulation and *ALMT1* expression in the roots. (A) Al content of CS and selected addition lines exposed to 25 μM Al for 48 h. Values are means ± SEM (n = 3). Asterisks indicate significant differences from CS (P<0.05, Fisher's PLSD test). (B) Al localization in root tips of CS, A, E, F, Hs and P. The roots were stained with Morin following exposure to 25 μM Al for 48 h. Bars = 200 μm. (C, D) RT-PCR analysis of *ALMT1* expression in the roots of CS, A, E, F, Hs and P non- exposed and exposed to 25 μM Al for 48 h. (Top) expression of *ALMT1*. (Bottom) expression of ribosomal 18S RNA gene used as internal control.

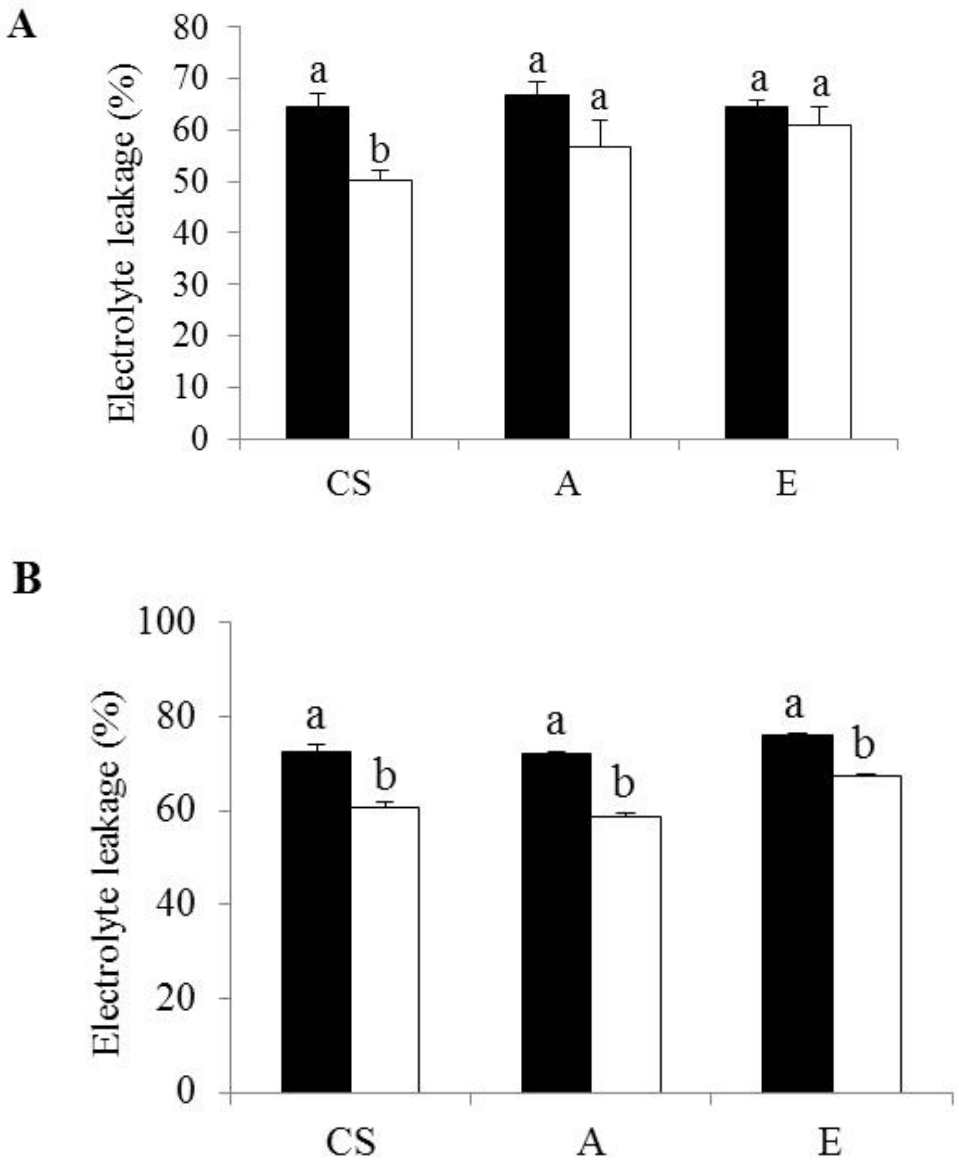


Fig. 1-6. Electrolyte leakage, indicator of the loss of plasma membrane integrity, of the roots of CS, A, and E after (A) 24 h and (B) 48 h at 0 (dark bars) or 25 μM Al (white bars). Values are means ± SEM (n = 4). Each replication included 4 replicated seedlings. Means with different letters differ significantly (P<0.05, Fisher's PLSD test).

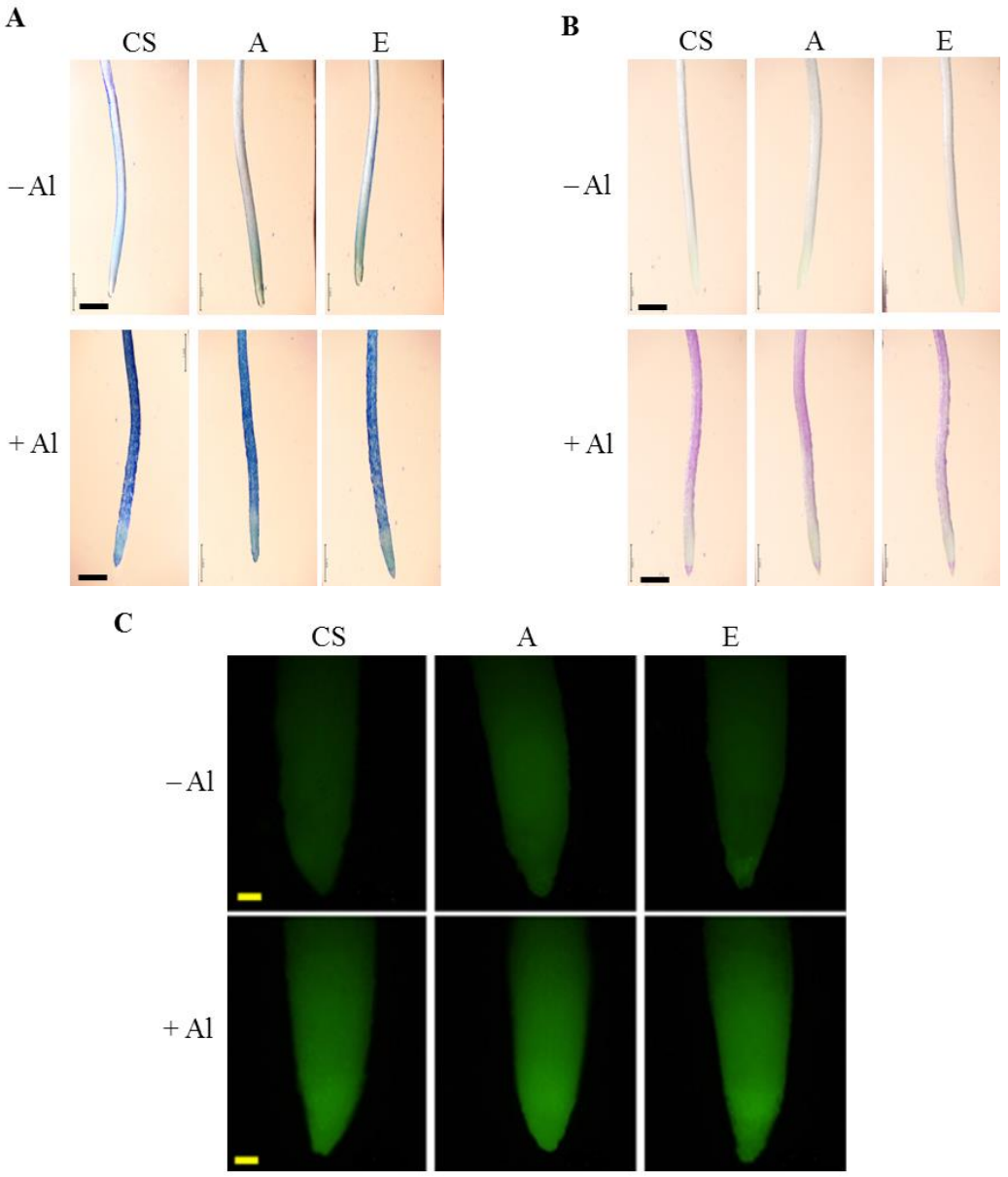


Fig. 1-7. Cell death, lipid peroxidation and distribution, and accumulation and distribution of H_2O_2 in root tips of CS, A, and E seedlings exposed to 25 µM Al for 48 h. Stained with (A) Evans blue to detect cell death, (B) Schiff's reagent to detect lipid peroxidation, or (C) DCF-DA to detect H_2O_2. Bars = 1 mm (A, B) or 200 µm (C).

Chapter 2

Impact of wheat-*Leymus racemosus* added chromosomes on wheat adaptation and tolerance to heat stress

2.1 Abstract

Adaptation of wheat (*Triticum aestivum* L.) to high temperatures could be improved by introducing alien genes from wild relatives. The responses of wheat-*Leymus racemosus* chromosome introgression lines to high temperature were examined to determine their potentiality for developing improved wheat cultivars. Introgression lines and their parent Chinese Spring were evaluated in a growth chamber at the seedling stage and in the field at the reproductive stage in two heat-stressed environments in Sudan. Optimum and late planting were used to ensure exposure of the plants to heat stress at the reproductive stage. The results revealed the impact of several *Leymus* chromosomes in improving wheat adaptation and tolerance to heat. Three lines possessed enhanced adaptation, whereas two showed high heat tolerance. Two addition lines showed a large number of kernels per spike, 32while one possessed high yield potential. Grain yield was correlated negatively with the heat susceptibility index, days to heading and maturity, and positively with kernel number per spike and triphenyl tetrazolium chloride assay under late planting. The findings suggest that these genetic stocks could be used as a bridge to introduce the valuable *Leymus* traits into a superior wheat genetic background, thus helping maximize wheat yield in heat-stressed environments.

2.2 Introduction

Wheat is the most important staple food for the majority of the world's population. Due to the current rapid exponential growth of the world population, an urgent expansion of the wheat production area and an increase in wheat productivity by unit area is therefore needed. Biotic and abiotic stresses and nutrient deficiencies are the biggest constraints of crop production.

Heat stress is considered one of the major factors limiting wheat production in tropical and subtropical environments. In these areas, high temperatures are known to affect crop development at all stages, imposing morphological and physiological changes that result in considerable grain yield loss (Al-Khatib and Paulsen 1990, Tahir *et al*. 2005, 2006, Tewolde *et al.* 2006). The optimum temperature for wheat growth and yield is within the range 18–24ºC. Stone and Nicolas (1994) reported that even a short period (5–6 days) of exposure to temperatures of 28–32ºC can result in significant decreases in yield of 20% or more. All plant processes are sensitive to and can be irreversibly damaged by heat. Elevated temperatures accelerate senescence, diminish the viable leaf area duration and reduce photosynthetic activities (Harding *et al.* 1990, Nagarajan *et al*. 1998). Heat stress also affects thylakoid membranes and leads to a loss in the number of chloroplasts per cell (Hurkman and Tanaka 1987). Accordingly, development of heat-tolerant cultivars is of major concern in wheat breeding programs.

Yield and its components are used widely as criteria for determining the heat tolerance of wheat (Reynolds *et al.* 1994, Khana-Chopra and Viswanathan, 1999). Although expensive to obtain, they are crucial measures of productivity in stressful

environments and highly applicable for comparing cultivars (Reynolds *et al*. 1994, Hede *et al.* 1999). Chlorophyll content (Alkhatib and Paulsen 1990), chlorophyll fluorescence (Smillie and Hethering 1983, Moffate *et al*. 1990) and triphenyl tetrazolium chloride cell viability assay (Porter *et al.* 1994) are among the physiological parameters used widely to evaluate heat stress tolerance in wheat.

The production and release of modern wheat cultivars after the green revolution enabled the expansion of wheat production in areas that experience heat stress, such as West Asia and North Africa. Despite the success of these cultivars, we remain far from solving the global food problem. To achieve a breakthrough in wheat breeding for stress tolerance and nutrient deficiency, development of new technologies and ideas is required. One of the possible ways is to discover and explore novel genes existed within the wheat gene pool, especially, the wild wheat relatives proved to be valuable gene sources for several important traits and contribute to increasing the available genetic diversity for development of new cultivars.

Due to the importance of *Leymus* as a novel source of useful traits, this study aimed to determine the response of wheat-*Leymus racemosus* introgression lines to heat stress and examine their potentiality to develop improved cultivars. The results indicated the presence of genotypic variability within addition lines for heat stress tolerance and revealed the potentiality of utilization of the genetic stocks identified in this study for improving wheat adaptation and heat stress tolerance.

2.3 Material and methods

2.3.1 Plant materials

I examined 12 wheat–*Leymus racemosus* chromosome additions and two substitution lines in the background of wheat cultivar Chinese Spring (CS) and their wheat background CS (Table 2-1). Hereafter the addition and substitution lines are designated together as 'introgression lines'. All lines and their wheat parent CS were provided by the Tottori Alien Chromosome Bank of Wheat (TACBOW) supported by National BioResource Project–Wheat, NBRP-wheat.

2.3.2 Growth chamber evaluation

The growth chamber experiment was carried out in a chamber in the Arid Land Research Center, Tottori, Japan. The seeds listed in Table 2-1 were sown directly in pots containing 1.00 kg organic soil under fully controlled conditions with a 22/18ºC day/night temperature, 40/50% day/night relative humidity, a 14/10 h (day/night) photoperiod and 80000 lux light intensity. One single seedling was maintained in each pot by clipping the secondary and tertiary tillers to eliminate the confounding effect of tiller number on plant responses under heat stress (Wardlaw 2002). Lines were grown in a completely randomized design with three replications for both the control condition and stressed condition. After 21 days, plants in the heat treatment group were exposed to high temperatures by transferring them to a controlled chamber set at 34/25ºC for 4 days. Heat-stressed plants were watered daily while those in the optimum temperature group were watered every other day. Pots were randomized every day to minimize spatial effects. Data were collected after 4 days of heat treatment on chlorophyll fluorescence and triphenyl tetrazolium chloride (TTC) cell viability assay. The chlorophyll fluorescence was measured in both the control and heat treated plants using MINI-PAM, a photosynthesis yield analyzer (WALZ, Germany).

TTC assay was carried out as described by Fokar *et al.* (1998) after 4 days heat acclimatization. Chlorophyll fluorescence data represent percentage of reduction from control and TTC assay data represent relative values to the control.

2.3.3 Field evaluation

In the first season (2011/2012), experiments were conducted at the Biotechnology and Biosafety Research Center (BBRC) experimental field, Shambat, Khartoum North, Sudan (15°32′N, 32°32′E), and in the second season (2012/2013), at Gezira Research Station experimental field, Agricultural Research Corporation (ARC), Wad Medani, Sudan (14º24′N, 33º29′E). Two planting dates were used in each field: an optimum planting date (2^{nd} week of November) and late planting date (2^{nd} week of December) to enable and insure exposure of the crop to heat stress during the grain filling period. Meteorological data, in particular, the weekly maximum, minimum and mean temperatures, during the two cropping seasons were obtained from meteorological stations located at the two experimental sites (Fig. 2-2A, B).

Seeds were sown manually in rows 0.2 m apart in plots consisting of 2 rows of 0.5 m length, in both seasons and locations. The seed rate was 10 g/m^2. Seeds were dressed with an insecticide Gaucho, (Imidacloprid 35% WP) (Bayer CropScience, USA) to control pests, especially termites and aphids. Triple superphosphate (4.3 g/m^2 of P_2O_5), was applied prior to planting by furrow placement and urea was applied before the second irrigation (8.6 g/m^2 of N). Irrigation was carried out at 7 to 10-day intervals to avoid water stress. Weeding was performed manually at least twice. All experiments were arranged in randomized complete block design with three replications.

Traits measured, their definitions and abbreviations are listed in Table 2-2. Grain yield was estimated per the main culm in the first season in Shambat due to lodging of some plots, whereas in Gezira lodging did not occur, and therefore, grain yield was estimated per plot. The heat susceptibility Index (HSI) was calculated according to the formula of Fischer and Maurer (1978): HSI = (1-Y/Yp)/D, where Y is the yield of the genotype at late planting, Yp is the mean yield of the genotypes at optimum planting and D (stress intensity) = 1-X/Xp, where X is the mean Y of all genotypes and Xp is the mean Yp of all genotypes. Genotypes were rated as highly tolerant (HSI $\leq$ 0.50), moderately tolerant (0.50<HSI$\leq$1.00) or susceptible (HSI>1.00) to high temperature (Fischer and Maurer 1978, Khanna-Chopra and Viswanathan 1999).

2.3.4 Statistical analysis

All data were subjected to analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test at $P<0.05$ using StatView software v. 5.0.1 (SAS Institute, Inc., USA). Field evaluation data were analyzed separately for each season then combined after testing the error mean squares for homogeneity. Simple correlation coefficients among all traits were calculated based on the overall means of the genotypes in the second season in Gezira.

2.4 Results

2.4.1 Evaluation under growth chamber conditions

Although a reduction in chlorophyll fluorescence was observed in CS and all tested lines, most of the introgression lines (except TAC11, TAC 3 and TAC5) exhibited a significantly ($P<0.05$) lower reduction in chlorophyll fluorescence compared to CS (Fig. 2-

1A). The substitution line TAC11 showed the highest reduction in chlorophyll fluorescence, while addition lines TAC3 and TAC5 showed a comparable reduction to CS. Addition line TAC9 did not experience a reduction in chlorophyll fluorescence, but rather showed a negative value, indicating that chlorophyll fluorescence under heat stress was enhanced in this line than the control condition. Addition lines TAC1, TAC6, TAC9 and TAC12 and substitution line TAC17 showed significantly higher values of TTC than CS, whereas all other lines had comparable values to CS (Fig 2-1B). These results indicate the presence of genotypic variability among the chromosome introgression lines for heat stress tolerance, and moreover, the impacts of *Leymus* chromosomes on wheat heat stress tolerance.

2.4.2 Field evaluation

Since the growth chamber experiments indicated genotypic variability in heat stress tolerance among the introgression lines, they were further tested in the field under heat stress conditions in Shambat and Gezira, Sudan. Maximum and average temperatures were higher in Gezira in the second season compared to Shambat in the first season (Fig. 2-2A, B). In general, the temperature from the 9^{th} to the 12^{th} week was lower than that from the 1^{st} to the 8^{th} week, and the 13^{th} to the 22^{nd} week.

In Shambat in the first season, heading of the optimum planting occurred between the 9^{th} to the 13^{th} week, while physiological maturity occurred from the 13^{th} to the 17^{th} week. For the late planting, heading occurred from the 12^{th} to the 16^{th} week and physiological maturity from the 16^{th} to the 19^{th} week (Fig. 2-2A). In Gezira, heading under optimum planting was observed from the 9^{th} to the 15^{th} week and physiological maturity from the

14th to the 18th week. Under late planting, heading was from the 13th to the 18th week and physiological maturity was from the 17th to the 20th week (Fig. 2-2B).

2.4.2.1 Heading and maturity

Heading was observed in CS between 90 to 97 days after sowing, while maturation occurred within 109 to 121 days in both seasons in Shambat and Gezira. The introgression lines exhibited a wide range of heading and maturity. Addition lines TAC1, TAC12 and TAC13 showed early heading within 58 to 66 days and matured within 88 to 103 days (Table 2-3). TAC15 and TAC17, on the other hand, were the latest to head and mature among all the introgression lines. Their heading was almost later than CS, while maturity was nearly comparable to CS, although TAC15 matured later than CS under optimum planting in Gezira. The remaining introgression lines showed earlier heading and maturity than CS. Except for the early genotypes (TAC1, TAC12 and TAC13); all genotypes were subjected to high temperatures during the grain filling stage under both optimum and late planting. The early genotypes experienced high temperatures during the grain filling stage under late planting. Moreover, heat stress occurred late in the season, reduced the mean DH and DM by 3 and 4 days, respectively. The effects of genotype (G) and planting date (PD) were significant for both traits in Shambat and Gezira (Table 2-3), while the effect of G x PD was significant for both traits in Gezira but not Shambat. This significant G x PD interaction and the reduction in DH and DM with late planting indicate the effect of environment on each genotype.

2.4.2.2 Plant height and tiller number

The mean plant height (PH) was higher in Shambat than Gezira under both optimum and late planting (Table 2-4). This could be attributed to the high temperature in Gezira at the beginning of the season (Fig. 2-2A, B), affecting plant development at an early stage. An apparent reduction in PH under late planting was observed in Gezira due to the higher temperatures (Table 2-4, Fig. 2-2A, B). In Shambat, the height of CS was 88 cm under optimum and 90 cm under late planting, while in Gezira it was 80 and 62 cm, respectively. Addition lines TAC8 and TAC10 were taller than CS under both optimum and late planting in Shambat and Gezira, indicating the presence of genes associated with PH in these *Leymus* added chromosomes. All other lines were comparable to CS in Shambat, although in Gezira, TAC3, TAC4, TAC6 and TAC11 were taller than CS and TAC14 and TAC17 were shorter under both optimum and late planting (Table 2-4). Analysis of variance revealed a significant G effect. The effects of PD and G x PD were significant only in Gezira, which was hotter than Shambat (Table 2-4, Fig. 2-2).

Tiller number (TN) was estimated in Gezira in the second season. The TN of CS was 45 under optimum planting and 29 under late planting. The TN of the addition lines ranged from 57 in TAC6 to 24 in TAC15 under optimum planting and from 63 in TAC10 to 30 in TAC15 under late planting (Table 2-4). TAC6 and TAC10 had the highest TN, while TAC13 and TAC15 had the lowest. There was a reduction in TN under late planting and an apparent reduction was observed in CS, TAC3, TAC4, TAC5 and TAC11. The effects of G, PD and G x PD were significant (Table 2-4).

2.4.2.3 Spike length and number of spikelets per spike

The spike length (SL) of CS was 9.5 and 8.2 cm under optimum planting and 9.9 and 7.2 cm under late planting in Shambat and Gezira, respectively (Table 2-5). The SL of the introgression lines ranged from 7.6 cm in TAC6 to 11.2 cm in TAC13 under optimum planting and from 7.5 cm in TAC14 to 11.5 cm in TAC13 under late planting. Among the introgression lines, substitution line TAC11 and addition line TAC13 had the longest spikes, while addition lines TAC14 and TAC15 had the shortest (Table 2-5).

The spikelets per spike (SPS) of CS were 21 and 22 under optimum planting and 21 and 19 under late planting in Shambat and Gezira, respectively (Table 2-5). The SPS of the introgression lines ranged from 17 in TAC13 to 24 in TAC4 under optimum planting and from 15 in TAC13 to 22 in TAC3, TAC4, TAC11 and TAC14 under late planting. None of the addition lines had a higher SPS than CS across environments and PD, except TAC6, which had more SPS in Shambat under optimum planting. The highest reduction in SPS was recorded in Gezira under late planting (Table 2-5). The variation in SL and SPS among genotypes was highly significant in both environments (Table 2-5). The PD differences were significant only in Gezira and no significant G x PD interaction was observed.

2.4.2.4 Number of kernels per spike and 1000 kernel weight

Kernel number per spike (KS) was higher in Shambat than Gezira under optimum and late planting due to the high temperatures in Gezira (Table 2-6, Fig. 2-2A, B). The KS of CS was 55 and 37 under optimum planting in Shambat and Gezira, respectively, and 51 and 26 under late planting, respectively. The KS of the introgression lines ranged from 3 in TAC15 to 68 in TAC6 under optimum planting and from 6 in TAC17 to 58 in TAC6 and TAC10 under late planting in the two environments (Table 2-6). TAC6 and TAC10 had

significantly higher KS than CS under optimum planting in both environments (Table 2-6). This result indicates the existence of genes related to KS in these two *Leymus* added chromosomes. Under late planting, a significantly higher number of KS compared to CS were observed in TAC6 and TAC10 in Shambat and in TAC1 and TAC14 in Gezira (Table 2-6). TAC11, TAC13, TAC15 and TAC17 had the lowest KS among the introgression lines. The greatest reduction in KS was observed under late planting in Gezira because of the higher temperatures. Introgression lines varied greatly in the magnitude of reduction due to high temperatures late in the season, ranging from 23% in TAC13 to 78% in TAC5 compared to 30% in CS. KS showed highly significant variation among the genotypes in both environments under optimum and late planting (Table 2-6). PD differed significantly in Gezira, and the G x PD interaction was highly significant in both environments indicating the strong influence of environment on the trait.

The kernel weight (KW) of CS was 16.1 g under optimum planting and 13 g under late planting, while in the introgression lines KW ranged from 9.3 g in TAC15 to 26.3 g in TAC13 under optimum planting and from 14.6 g in TAC1 to 22.2 g in TAC13 under late planting (Table 2-6). TAC13 and TAC14 had higher KW than CS under optimum planting, whereas under late planting, TAC13, TAC4, TAC9 and TAC11 had higher KW than CS. The reduction in KW due to heat stress under late planting was 19% in CS, whereas the reduction in KW in the introgression lines ranged from no reduction in TAC4, TAC5, TAC6, TAC9 and TAC11 to 24% in TAC14. Genotypes differed significantly in KW under optimum and late planting. PD had no significant effect on KW; however, the interaction G x PD was significant, indicating the effect of high temperature on KW (Table 2-6).

2.4.2.5 Chlorophyll content, grain yield and heat susceptibility index

The chlorophyll content (ChC) of CS was 38.5 SPAD units under optimum planting and 38 SPAD units under late planting. The ChC of the introgression lines ranged from 35 SPAD units in TAC9 to 46.6 SPAD units in TAC13 under optimum planting and from 36.8 SPAD units in TAC10 to 45.9 SPAD units in TAC13 under late planting (Table 2-7). These results indicate the presence of genes associated with ChC in the *Leymus* chromosome added to TAC13. TAC9 had a lower ChC than CS under both optimum and late planting. Overall, the average chlorophyll content of the genotypes was higher under optimum planting than late planting. The G effect was significant and the influence of environment was revealed by the significant PD effect. The interaction G x PD was not significant (Table 2-7).

A reduction in GY due to the high temperature under late planting was observed in Shambat and Gezira. In Shambat, the GY of CS was 1.5 g/spike under optimum planting and 1.3 g/spike under late planting, whereas the introgression lines ranged from 0.6 g/spike in TAC17 to 2.3 g/spike in TAC6 under optimum planting, and from 0.6 g/spike in TAC17 to 1.9 g/spike in TAC10 under late planting. In Gezira, the GY of CS was 51 g/m^2 under optimum planting and 14 g/m^2 under late planting, whereas the introgression lines ranged from 10 g/m^2 in TAC15 to 265 g/m^2 in TAC14 under optimum planting and from 8 g/m^2 in TAC11 to 78 g/m^2 in TAC6 under late planting (Table 2-7). TAC1, TAC3, TAC6, TAC10, TAC12, TAC13 and TAC14 had significantly higher GY than CS under both optimum and late planting in Shambat and Gezira, although these genotypes suffered a high yield reduction due to the high temperature under late planting (Table 2-7). The high yields of

these genotypes indicate the presence of genes related to yield potential in these *Leymus* added chromosomes. TAC15 and TAC17 had lower GY compared to the other introgression lines and CS. In Gezira, TAC4, TAC5 and TAC9 had lower GY than CS under optimum planting, while under late planting their GY was comparable to CS (Table 2-7). The genotypes showed highly significant differences in GY in both environments among the PD. The effect of PD and the interaction G x PD were also highly significant (Table 2-7), indicating that the GY of the genotypes responded differently to the two PD.

Based on the heat susceptibility index (HSI), the indicator of GY reduction, TAC6 and TAC12 were classified as highly heat tolerant, showing HSI < 0.5 (Table 2-7). TAC1, TAC3, TAC10 and TAC13 were classified as moderately heat tolerant (1 > HSI > 0.5), whereas the rest of the addition lines and CS were classified as heat sensitive (HSI > 1) (Table 2-7) (Fischer and Maurer 1978, Khanna-Chopra and Viswanathan 1999). Overall, the genotypes showed highly significant variation in HSI.

2.4.2.6 Correlation between plant traits with grain yield and heat susceptibility index

The correlation between GY and the other plant traits was estimated from the means of all the genotypes within the growth chamber and under optimum and late planting in Gezira, where the highest reduction in several plant traits was observed under late planting. GY under optimum planting was correlated negatively with DH, DM and SL and positively with KS, KW and ChC. under late planting; GY was correlated negatively with DH and DM and positively with KS and TTC (Table 2-8). No correlation was observed between GY and HSI under optimum planting, but under late planting a highly significant negative

correlation was observed. HSI was correlated positively with DH and DM and negatively with KS and TTC under both optimum and late planting conditions (Table 2-8).

2.5 Discussion

The results clearly indicate the positive impact of some added *Leymus* chromosomes on several wheat traits and, revealing the value of these alien chromosomes on wheat heat stress tolerance.

The negative correlations between DH and DM with GY and their positive correlations with HSI indicate that early heading and maturity are preferable for high yield gain in heat-stressed environments. TAC1, TAC12 and TAC13 showed early heading and maturity coupled with a higher GY than CS under both the optimum and late planting conditions (Tables 2-3, 2-7). However, the GY of these lines was reduced under late planting. On the other hand, low yield was associated with late heading and maturity in TAC15 and TAC17 (Tables 2-3, 2-7), suggesting that the high yield of TAC1, TAC12 and TAC13 is attributed to their adaptation and ability to escape late heat stress. Early heading and maturity enabled these lines to fill their grains normally and escape the late heat stress occurring at the end of the season. Iqbal *et al.* (2007) reported early maturity resulted in avoidance of late-season frost damage during the short growing season in northern high latitudes in Canadian spring wheat. However, a grain yield penalty was detected in these early maturing cultivars (Iqbal *et al.* 2007).

Based on the HSI results, TAC1 and TAC13 were classified as moderately heat tolerant, and TAC12 as highly heat tolerant (Table 2-7). GY was strongly correlated with TTC under late planting conditions (Table 2-8). TAC12 had a significantly higher TTC

value than TAC1 and TAC13 (Fig. 2-2B), indicating efficient mitochondrial electron transport activity, and therefore we conclude that in addition to the avoidance, TAC12 also has a heat tolerance mechanism. Addition lines TAC1 and TAC12 and substitution line TAC17 harbor the same *Leymus* chromosome in homoeologous group 2 (HG2) (Larson *et al.* 2012). However, TAC17 showed delayed flowering and maturity compared to TAC1 and TAC12. This was attributed to the absence of the photoperiod-response *Ppd-B1* allele on chromosome 2B, which was replaced in TAC17 by chromosome 2Lr#1 of *L. racemosus* (Qi *et al.* 1997). TAC1 was developed by Kishii *et al*. (2004) in Japan, whereas TAC12 was developed by Qi *et al*. (1997) using different *L. racemosus* strains in China; therefore, the difference between the two in GY and heat tolerance could be attributed to allelic differences in the *L. racemosus* strains used to develop the two addition lines.

TAC13 has a longer spike than CS, but its SPS and KS were lower than those of CS (Tables 2-5, 2-6). These results indicate that the added chromosomes resulted in changes in spike morphology. TAC13 had a low number of grains; however, it had more GY as a result of its high ChC, giving a higher KW (Tables 2-6, 2-7). This high KW subsequently compensated for the loss in grain number. TAC13 harbors a *Leymus* chromosome from homoeologous group 5; QTL affecting KW in wheat are located on chromosomes 2B, 5B and 7A (Groos *et al*. 2003)

The higher GY of TAC6 and TAC10 compared to CS under both optimum and late planting (Table 2-7) was attributed to their higher TN and KS than CS under both conditions, as well as to their stable KW, since neither line suffered any reduction due to the high temperature under late planting (Tables 2-4, 2-6). Based on the HSI results, TAC6

was classified as highly heat tolerant and TAC10 as moderately heat tolerant (Table 2-7). This finding was attributed to the fact that TAC6 had a significantly higher TTC value than CS and TAC10, indicating higher mitochondrial efficiency under heat stress conditions (Fig. 2-1B). TAC6 harbors a *Leymus* chromosome from homoeologous group 5, while TAC10 harbors chromosomes from homoeologous group 3/7 (Kishii *et al.* 2004, Larson *et al.* 2012). Byrne *et al.* (2002) reported that loci on chromosomes 2B and 5B are the most important for heat tolerance. In addition, the above findings indicate the presence of genes related to TN and KS on these two *Leymus* added chromosomes.

TAC14 had the highest GY among the introgression lines under optimum planting in Gezira (Table 2-7). This was the result of its high TN and KW (Tables 2-4, 2-6). However, the GY of this line decreased drastically between optimum and late planting, and it was subsequently classified as heat sensitive based on the HSI results. The higher GY of this line under the favorable conditions of optimum planting suggest the presence of genes related to yield potential in the added *Leymus* chromosome.

Addition line TAC5 and substitution line TAC11 harbor the same *Leymus* chromosome from homoeologous group 3 (Kishii *et al.* 2004, Larson *et al.* 2012). TAC11 had a higher reduction in chlorophyll florescence than TAC5 (Fig. 2-2A), indicating the importance of wheat chromosome 2B, which was substituted in TAC11 by *Leymus* chromosome H, in reducing the effect of high temperature on PSII activity. On the other hand, TAC5 had more KS than TAC11 under the favorable conditions of optimum planting, indicating that wheat chromosome 2B includes genes that influence grain number. In

analysis of spring wheat populations for heat tolerance, loci on chromosomes 2B and 5B were found to be most important (Byrne *et al*. 2002).

The grain yield (GY) showed a highly significant correlation with HSI under late planting conditions (Table 2-8), indicating the validity of using HSI as a selection criteria for heat tolerance in wheat. Yang *et al.* (2009) also reported a highly significant correlation between the GY of hexaploid amphiploids and HSI under heat stress conditions. KS was positively correlated with GY and negatively with HSI under both optimum and late planting conditions (Table 2-8). This finding indicates the importance of KS as a determining factor of yield of the lines under heat stress. A correlation between GY and KS was previously reported in wheat under heat stress (Balla *et al*. 2012, Mohammadi *et al*. 2004). Here, TTC was correlated positively with GY and negatively with HSI under late planting conditions, indicating plants showing enhanced mitochondrial efficiency under heat stress also have better GY and lower HSI values. This result indicates that TTC assay could be used as a selection criterion for heat stress tolerance at the seedling stage. TTC assay is an efficient technique for quantification of acquired high temperature tolerance in wheat cultivars (Porter *et al.* 1994). Dhanda and Munjal (2006) previously reported a positive correlation between GY and TTC in 20 diverse wheat genotypes under normal and heat stress conditions.

Chlorophyll content (ChC) was correlated positively with GY under optimum planting, but not late planting (Table 2-8). Reynolds *et al*. (1994) suggested that sustained ChC during maturation was an efficient indicator of heat tolerance in wheat cultivars. Hede *et al.* (1999) also found a significant correlation between leaf chlorophyll content and

kernel weight in 2,255 Mexican landraces and revealed the reliability of chlorophyll level as a heat tolerance indicator in wheat. In contrast, Balla *et al*. (2012) did not find a correlation between ChC and GY or its components under heat stress conditions in a doubled haploid population derived from a cross between heat tolerant and heat sensitive wheat cultivars.

In conclusion, this study revealed the impact of certain *Leymus* chromosomes on enhancing wheat tolerance and adaptation to high temperatures. Chromosomes A, 2Lr#1 and 5Lr#1 added to TAC1, TAC12 and TAC13, respectively, enhanced adaptation, whereas chromosomes I and N in TAC6 and TAC10, respectively, enhanced grain number per spike and heat tolerance. The results also indicated the importance of chromosome 7Lr#1 added to TAC14 as a source of yield potential. CS, the parent of these addition lines, is a classic genotype, showing low yield potential. These genetic stocks could therefore be used as a bridge to introduce the valuable *Leymus* traits identified in this study into superior wheat backgrounds, thus enhancing wheat adaptation and maximizing yield potential under stressed environments.

Table 2-1. List of the wheat-*Leymus* chromosome introgression lines used in this study, their strain, chromosome name and homoeologous groups.

Strain ID	Abbreviation	Strain name and chromosome name	Homoeologous group	Number of chromosomes	Reference
TACBOW0001[a]	TAC1	*Leymus racemosus* A addition	2	44	Kishii *et al.* 2004
TACBOW0003	TAC3	*L. racemosus* E addition	ND[b]	44	Kishii *et al.* 2004
TACBOW0004	TAC4	*L. racemosus* F addition	4	44	Kishii *et al.* 2004
TACBOW0005	TAC5	*L. racemosus* H addition	3	44	Kishii *et al.* 2004
TACBOW0006	TAC6	*L. racemosus* I addition	5	44	Kishii *et al.* 2004
TACBOW0008	TAC8	*L. racemosus* k addition	6	44	Kishii *et al.* 2004
TACBOW0009	TAC9	*L. racemosus* l addition	2	44	Kishii *et al.* 2004
TACBOW0010	TAC10	*L. racemosus* n addition	3,7	44	Kishii *et al.* 2004
TACBOW0011	TAC11	*L. racemosus* H substitution	3	42	Kishii *et al.* 2004
TACBOW0012	TAC12 (NAU516)	*L. racemosus* 2Lr#1 addition	2	44	Qi *et al.* 1997
TACBOW0013	TAC13 (NAU504)	*L. racemosus* 5Lr#1 addition	5	44	Qi *et al.* 1997
TACBOW0014	TAC14 (NAU501)	*L. racemosus* 7Lr#1 addition	7	44	Qi *et al.* 1997
TACBOW0015	TAC15 (NAU501)	*L. racemosus* 7Lr#1 addition	7	44	Qi *et al.* 1997
TACBOW0017	TAC17 (NAU551)	*L. racemosus* 2Lr#1 substitution	2	42	Qi *et al.* 1997
Chinese Spring	CS				

[a]TACBOW: Tottori Alien Chromosome Bank of Wheat supported by NBRP-wheat; [b]ND, not determined.

Table 2-2. Traits measured their abbreviations and definitions.

Trait	Abbreviation	Definition
Days to heading	DH	Days from sowing to ear emergence.
Days to physiological maturity	DM	Days from sowing to physiological maturity (loss of green color from the glumes).
Plant height	PH	Height from the ground to the tip of the spike.
Tiller number	TN	Number of culms with spikes per each plant or in a specific area within the plot.
Spike length	SL	Length from the base to the tip of the spike, measured at physiological maturity.
Number of spikelets per spike	SPS	Number of florets per spike, measured at physiological maturity.
Kernel number per spike	KS	Number of kernels counted after reaching physiological maturity, measured by counting the number of seeds after threshing each spike individually by hand.
1000 kernel weight	KW	Weight of 1000 kernels taken from each plant in each replication, weighed on a sensitive electronic balance.
Grain yield	GY	Yield after field maturity and drying (a loss of chlorophyll from all plants).
Chlorophyll content	ChC	The total amount of green pigment in the flag leaf during the mid-grain filing stage, estimated in SPAD units using a chlorophyll meter (SPAD-502, Minolta, Japan).

Table 2-3. Days to heading and maturity of the addition lines and their parent CS during evaluation for heat stress tolerance at optimum planting (OP) and late planting (LP) in Shambat (2011/2012) and Gezira (2012/2013), Sudan.

	Days to heading				Days to maturity			
	Shambat		Gezira		Shambat		Gezira	
Line	OP	LP	OP	LP	OP	LP	OP	LP
TAC1	58	62	62	64	96	91	98	91
TAC3	83	84	79	80	110	108	103	100
TAC4	80	86	81	84	113	108	109	112
TAC5	82	87	85	84	113	108	112	116
TAC6	84	86	78	79	112	108	100	104
TAC8	80	85	77	74	110	106	105	99
TAC9	82	82	92	76	114	107	115	106
TAC10	76	80	81	77	110	103	105	109
TAC11	78	81	81	78	110	106	105	106
TAC12	61	63	66	64	97	94	103	92
TAC13	58	61	64	60	90	88	100	89
TAC14	71	78	72	72	103	101	101	98
TAC15	103	101	107	97	117	111	129	108
TAC17	95	97	107	113	126	115	NA[d]	NA
CS	97	90	96	93	121	113	114	115
Mean	79	82	82	79	109	104	107	103
G[a]	***	***	***	***	***	***	***	***
LSD	5.873	4.613	3.090	3.680	9.500	3.687	4.030	4.360
SE (G)	2.0	1.7	2.0	2.0	1.5	1.2	1.3	1.3
PD[b]		**		***		***		***
G x PD		NS[c]		***		NS		***
CV %	16	14	17	17	9	7	8	8

[a]G, genotype; [b]PD, planting date; **, *** Significant at 0.01 and 0.001 probability levels, respectively; [c]NS, not significant; [d]NA, not available.

Table 2-4. Plant height (PH) and tiller number (TN) of the addition lines and their parent CS during evaluation for heat stress tolerance at optimum planting (OP) and late planting (LP) in Shambat (2011/2012) and Gezira (2012/2013), Sudan.

	Plant height (cm)				Tiller number	
	Shambat		Gezira		Gezira	
Line	OP	LP	OP	LP	OP	LP
TAC1	95	95	65	70	44	44
TAC3	92	92	92	78	54	42
TAC4	89	94	95	72	47	30
TAC5	82	90	85	65	46	21
TAC6	97	100	93	72	57	58
TAC8	101	105	88	73	43	44
TAC9	83	87	73	60	34	42
TAC10	102	103	88	73	56	63
TAC11	102	92	92	77	50	31
TAC12	99	93	68	65	38	43
TAC13	96	92	63	58	34	31
TAC14	93	85	70	53	54	57
TAC15	96	91	62	65	24	30
TAC17	91	97	60	53	39	NA[d]
CS	88	90	80	62	45	29
Mean	94	94	78	66	44	40
G [a]	**	***	***	***	***	***
LSD	10.81	5.48	4.972	7.024	9.7	12.24
SE (G)	1.23	0.9	2	1.28	1.55	2
PD [b]		NS [c]		***		**
G x PD		NS		***		***
CV%	8	6.5	16.4	13	23	33

[a]G, genotype; [b]PD, planting date; **, *** Significant at 0.01 and 0.001 probability levels, respectively; [c]NS, not significant; [d]NA, not available.

Table 2-5. Spike length (SL) and number of spikelets per spike (SPS) of the addition lines and their parent CS during evaluation for heat stress tolerance at optimum planting (OP) and late planting (LP) in Shambat (2011/2012) and Gezira (2012/2013), Sudan.

	Spike length				Spikelet/spike			
	Shambat		Gezira		Shambat		Gezira	
Line	OP	LP	OP	LP	OP	LP	OP	LP
TAC1	9.6	9.6	8.6	7.9	18	19	19	19
TAC3	9.2	9.2	8.3	7.9	22	22	22	19
TAC4	9.2	9.2	8.9	8.6	22	22	24	21
TAC5	9.7	9.1	9.2	7.7	21	20	22	19
TAC6	9.6	9.5	7.6	7.7	23	21	19	18
TAC8	9.8	10.0	8.7	8.4	20	22	18	18
TAC9	9.8	9.9	10.4	8.9	21	21	22	17
TAC10	9.1	9.3	8.6	8.0	22	21	21	19
TAC11	11.1	10.9	10.2	10.1	21	22	20	19
TAC12	8.9	8.9	8.2	7.9	20	19	20	19
TAC13	11.2	11.5	9.2	8.4	17	17	19	15
TAC14	7.7	7.5	6.9	5.7	21	22	22	20
TAC15	8.3	8.2	7.9	NA	20	20	18	NA
TAC17	10.2	9.8	NA	6.8	20	19	NA[d]	16
CS	9.5	9.9	8.2	7.2	21	21	22	19
Mean	10	9	9	8	20	21	21	19
G[a]	***	***	***	***	***	***	**	***
LSD	0.724	0.734	1.217	1.204	1.833	1.994	3.191	1.826
SE	0.128	0.134	0.15	0.183	0.235	0.251	0.383	0.227
PD[b]		NS		**		NS		***
G x PD		NS[c]		NS		NS		NS
CV%	10	10	11	15	9	9	12	9.7

[a]G, genotype; [b]PD, planting date; **, *** Significant at 0.01 and 0.001 probability levels, respectively; [c]NS, not significant; [d]NA, not available.

Table 2-6. Kernel number per spike (KS) and 1000 kernel weight (KW) of the addition lines and their parent CS during evaluation for heat stress tolerance at optimum planting (OP) and late planting (LP) in Shambat (2011/2012) and Gezira (2012/2013), Sudan.

	Kernel number/spike				1000 kernel weight (g)	
	Shambat		Gezira		Gezira	
Line	OP	LP	OP	LP	OP	LP
TAC1	53	50	49	32	18.6	14.6
TAC3	54	57	40	24	17.3	15.7
TAC4	43	48	16	8	15.7	16.8
TAC5	44	43	38	8	13.5	16.4
TAC6	68	58	47	29	15.6	16.5
TAC8	53	53	29	16	16.9	16.0
TAC9	39	41	29	19	14.1	18.8
TAC10	59	58	45	26	16.5	16.5
TAC11	32	38	28	9	14.9	16.7
TAC12	54	49	36	27	19.1	16.5
TAC13	43	43	32	14	26.3	22.2
TAC14	49	57	47	36	21.4	16.3
TAC15	45	38	3	NA	9.3	NA
TAC17	8	6	NA[d]	36	NA	NA
CS	55	51	37	26	16.1	13.0
Mean	47	46	34		16.8	16.6
G[a]	***	***	***	***	***	*
LSD	4.008	6.706	8.207	4.915	3.372	3.063
SE (G)	1.807	1.717	2	1.52	0.643	0.431
PD[b]		NS[c]		***		NS
G x PD		***		***		*
CV %	29	28	38	44	24	16.0

[a]G, genotype; [b]PD, planting date; *, **, *** Significant at 0.05, 0.01 and 0.001 probability levels, respectively; [c]NS, not significant; [d]NA, not available.

Table 2-7. Chlorophyll content (ChC) at the mid grain filling stage, grain yield and the heat susceptibility index (HSI) of the addition lines and their parent CS during evaluation for heat stress tolerance at optimum planting (OP) and late planting (LP) in Shambat (2011/2012) and Gezira (2012/2013), Sudan.

	Chlorophyll content*		Grain yield				
	Gezira		Shambat (g/plant)		Gezira (g/m^2)		
Line	OP	LP	OP	LP	OP	LP	HSI
TAC1	44.8	44.4	1.9	1.7	122	56	0.59
TAC3	44.8	41.3	1.8	1.8	102	39	0.93
TAC4	39.8	42.5	1.3	1.4	12	17	1.35
TAC5	40.9	40.5	1.6	1.2	25	15	1.38
TAC6	41.3	39.5	2.3	1.7	132	78	0.24
TAC8	43.7	41.5	2.0	1.5	73	9	1.50
TAC9	35.0	37.6	1.4	1.3	11	14	1.41
TAC10	43.2	36.8	2.1	1.9	133	50	0.71
TAC11	42.7	40.9	1.4	1.1	53	8	1.51
TAC12	43.4	41.9	2.1	1.7	121	74	0.25
TAC13	46.6	45.9	1.9	1.8	110	56	0.59
TAC14	44.5	42.3	2.1	1.9	265	29	1.12
TAC15	46.3	44.2	1.4	0.9	10	NA	NA
TAC17	42.4	39.7	0.6	0.6	NA[d]	NA	NA
CS	38.5	38.0	1.5	1.3	51	14	1.40
Mean	42.5	41.1	2	1	87	35	1
G[a]	***	***	***	***	***	***	***
LSD	3.296	4.682	0.243	0.359	25.15	6.95	0.310
SE (G)	0.51	0.57	0.059	0.056	10.62	3.95	0.063
PD[b]		***		***		***	
G x PD		NS[c]		***		***	
CV %	8	9.3	27	29	77	69	41

* Chlorophyll content is indicated by SPAD unit; [a]G, genotype; [b]PD, planting date; *** Significant at 0.001 probability level. [c]NS, not significant; [d]NA, not available.

Table 2-8. Correlation coefficients of grain yield and HSI with other plant traits measured in the addition lines and their parent CS during evaluation for heat stress tolerance in a growth chamber and the field under optimum planting (OP) and late planting (LP) in Gezira, Sudan, in the 2012/ 2013 season.

	Grain yield		HSI	
Trait	OP	LP	OP	LP
Days to heading	-0.54*	-0.62*	0.64*	0.57*
Days to maturity	-0.7**	-0.63*	0.69**	0.58*
Plant height	-0.35	-0.08	0.33	0.005
Tiller number	0.42	0.46	-0.08	-0.49
Spike length	-0.75**	-0.27	0.44	0.23
Spikelets per spike	-0.20	-0.20	0.39	0.23
Kernel number per spike	0.69**	0.55*	-0.55*	-0.54*
1000 Grain weight	0.58*	0.18	-0.45	-0.17
HSI	-0.43	-0.99***	1.00	1.00
Chlorophyll content	0.59*	0.31	-0.45	-0.25
Chlorophyll fluorescence	0.49	0.50	-0.32	0.5
TTC	0.11	0.71**	-0.68**	-0.68**

*, **, *** Significant at 0.05, 0.01 and 0.001 probability levels, respectively.

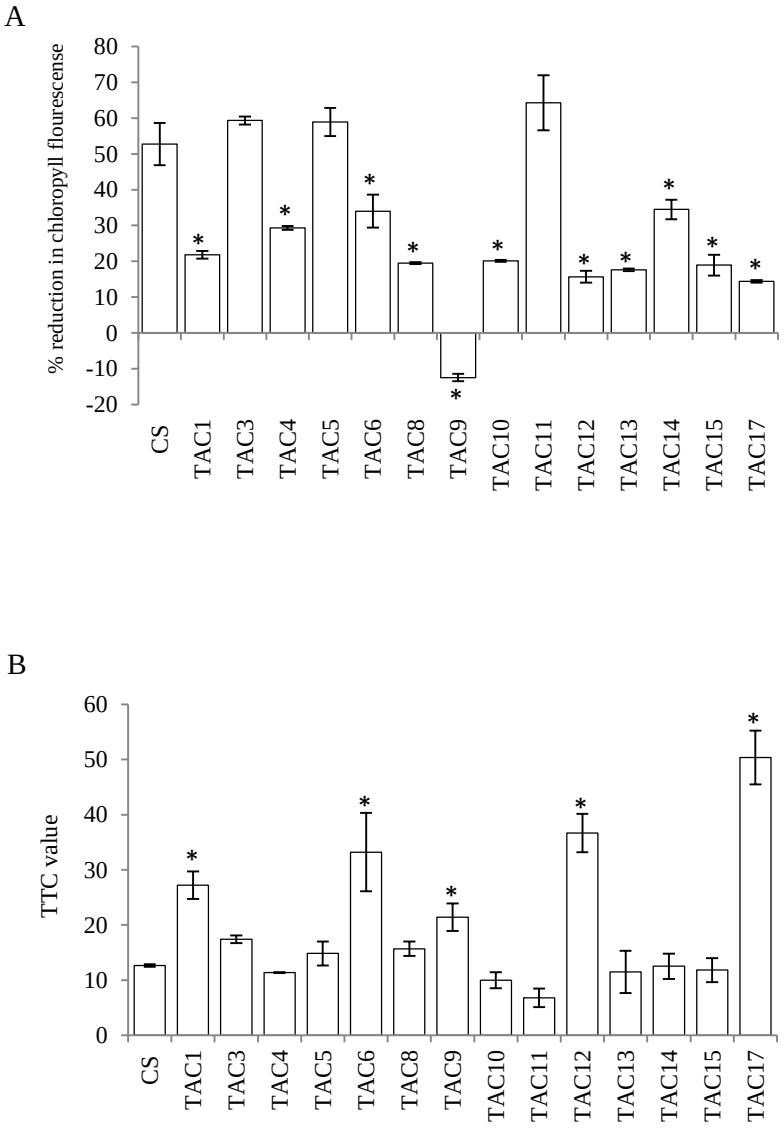


Fig. 2-1. Percentage of reduction in chlorophyll fluorescence (A) and triphenyl tetrazolium chloride (TTC) cell viability assay values (B) in the introgression lines and their parent CS in a growth chamber under normal (22/18ºC) and heat stress (34/25ºC) conditions. Asterisks denote significant differences from CS (P <0.05, Fisher's PLSD test).

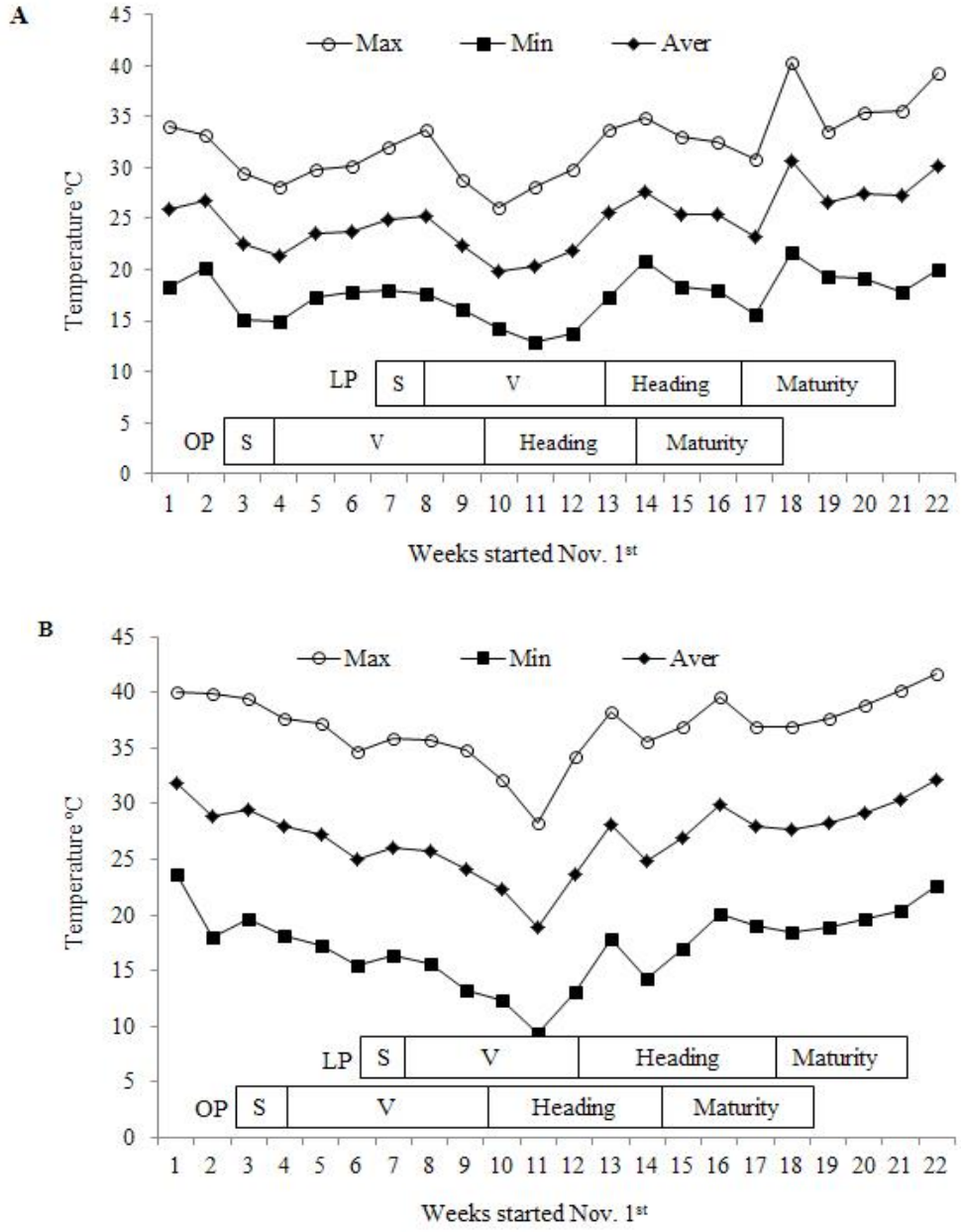


Fig. 2-2. Weekly maximum, minimum and average temperatures at (A) Shambat during the 2011/2012 cropping season and (B) Gezira during the 2012/2013 season. Time ranges of heading and maturity are indicated. OP and LP denote optimum planting and late planting, respectively. Time ranges S and V denote sowing and vegetative growth, respectively

Chapter 3

An insertion in the promoter of wheat-*Leymus racemosus* introgression lines is responsible for early flowering

3.1 Abstract

Vernalization genes determine the winter/spring growth habit in wheat and play decisive roles in adaptation to a wide range of environments, and in yield potential in stressed environments. In a previous study of heat stress tolerance using 15 introgression lines, we found three lines (TAC1, TAC13 and TAC17) that flowered a month earlier than their wheat background Chinese Spring (CS) with the dominant *Vrn-D1* gene. It is hypothesized that the vernalization requirement of CS was cancelled in the introgression lines, and this cancellation led to the early flowering. This study describes by what genomic changes do those lines flower earlier than CS. Specific molecular marker analysis revealed that these lines had the dominant *Vrn-A1* allele, whereas CS had the winter recessive *vrn-A1* allele. Unlike the CS winter allele, in the promoter region, the spring *Vrn-A1* allele of the introgression lines had a large insertion of 220-bp and a small insertion of 131-bp. Sequence analysis indicates that the large insertion of the introgression lines is similar to the insertion of the spring genotype Triple Dirk D (TDD). The insertion of TAC1 showed 1 SNP (T/G), whereas TAC17 showed an extra nucleotide (T) in addition to 1 SNP (T/C). The small insertion of the introgression lines differs from that of TDD by insertion, deletion and inversion events. Results of molecular marker analysis exclude the possibility of outcrossing and suggested that this insertion is due to genetic event occurred during the maintenance of the introgression lines.

3.2 Introduction

Wheat (*Triticum aestivum* L.) is one of the most widely grown food crops in the world, and grows under a wide range of environmental conditions. This wide adaptability is largely controlled by three groups of genetic factors (Kato and Yamagata, 1988): vernalization genes, *Vrn*, which determine a plant's vernalization requirement, photoperiod genes, *Ppd*, which determine its photoperiod sensitivity, and genes that control earliness per se. These groups act together to determine flowering time and therefore define the genotype adaptability to particular environmental conditions and the resulting yield potential (Worland *et al.* 1998; Gororo *et al.* 2001).

Vernalization genes determine growth habits, which divide wheat into winter and spring classes. Winter cultivars are mainly adapted to areas with average January temperature between –7 and 4°C, whereas spring cultivars are adapted to areas with temperatures above this range (Iwaki *et al.* 2000, 2001). Spring types result from mutation of the Vernalization-1 (*Vrn-1*), *Vrn-2* or *TaFT1* (also called *Vrn-3*) genes (Yan *et al.* 2003, 2004a, 2004b, 2006, Fu *et al.* 2005). *Vrn-1* encodes a MADS-box transcription factor whose expression increases quantitatively during vernalization. This promotes flowering by inhibiting expression of the flowering repressor *Vrn-2* (Trevaskis *et al*. 2006, Distelfeld *et al.* 2009). Semi-dominant mutations at one or more of the homoeoallelic *Vrn-A1*, *Vrn-B1* or *Vrn-D1* genes (chromosomes 5A, 5B and 5D, respectively) are the predominant cause of spring types in wheat (Fu *et al.* 2005). The cloning of wheat vernalization genes (Yan *et al.* 2003, 2006) and the identification and characterization of alleles for spring and winter growth habits (Yan *et al*. 2004a, 2004b, Fu *et al*. 2005) have facilitated the development of

gene-specific markers and has enabled the screening of large collections of wheat germplasm with allelic diversity in the *Vrn-1* genes.

Several reports described the *Vrn-1* allelic composition of wheat germplasm and revealed its effects on grain yield potential under various environmental conditions (for example, Iqbal *et al*. 2007). These reports clearly indicated the decisive role of the *Vrn-1* allelic composition in regional adaptation and agronomic performance of wheat genotypes. Owing to climate change and predicted increases in the global temperature, which may lead to short and warm growing season, early flowering may become advantageous for high yield and stable food production. Therefore, optimizing the *Vrn* allelic composition for specific production environments may offer possibilities for developing spring wheat cultivars with higher yield potential.

Wheat's ancestors and many modern varieties are characterized as photoperiod sensitive. They flower rapidly in long days but are late flowering in short days. On the other hand, day neutral (photoperiod insensitive) varieties flower rapidly in short or long days. This enables production in environments where appropriate temperature and rainfall coincide with short day conditions or where early flowering avoids high summer temperatures, as in Southern Europe (Worland and Snape 2001). The day neutral phenotype was an important component of the 'Green Revolution' and continues to be widely used globally. It results from semi-dominant mutations at one or more of the collinear (homoeoallelic) Photoperiod-1 (*Ppd-1*) loci on chromosomes 2A, 2B and 2D. Four day-neutral alleles have been characterized in wheat previously. They are given a suffix (*Ppd-A1a*, *Ppd-B1a* and *Ppd-D1a*) while wild type alleles have a *b* suffix (Mclntosh *et al.* 2003).

Two *Ppd-A1a* alleles and the single *Ppd-D1a* allele are deletions that remove a shared region of the promoter likely to be important for regulation (Beals *et al.* 2007, Wilhelm *et al.* 2009). In addition to the *Vrn-1* and *Ppd-1* mutant alleles that having sequence change such as insertions, deletions or point mutations, copy number variations has been reported to be another source of alteration of flowering time in wheat (Diaz *et al.* 2012).

Chapter 2 describes the impact of several *Leymus* chromosomes on wheat heat stress tolerance. The results indicated that four introgression lines were earlier in their heading and maturity than the background cultivar, Chinese Spring (CS). Moreover, grain yield and heat susceptibility index were highly correlated with days to heading and days to maturity. I hypothesized that the vernalization requirement of CS was cancelled in the introgression lines, and this cancellation led to the early flowering. This study was intended to describe by what genomic changes do those lines flower earlier than CS?

3.3 Materials and methods

3.3.1 Plant materials and phenotypic evaluation

Four wheat-*Leymus racemosus* introgression lines (TAC1, TAC13 and TAC17) and their wheat background Chinese Spring (CS) in addition to Triple Dirk D (TDD) line were used in this study. Triple Dirk D (TDD) line was kindly provided by Prof. K. Kato from Okayama University. Phenotypic evaluation was carried in the field of the Arid Land Research Center, Tottori University (ARLC, Tottori, Japan; 35°32′N, 134°13′E), and in the growth chamber under long day condition (16 h) without vernalization at 25°C during the day, 15°C during the night, and 60% relative humidity.

3.3.2 DNA extraction and molecular markers analysis

Genomic DNA was extracted from the leaves of 10 days-old seedlings using the CTAB method described by Clarck (1997). Specific primer pairs were used to identify allelic variation of *Vrn-A1, Vrn-B1, Vrn-D1*, and *Vrn-B3* (Yan *et al.* 2004a, 2006, Fu *et al.* 2005) (Table 3-1). To exclude the effect of differences in flowering time among the accessions due their photoperiod genes, we used the primer pairs, Ag5del_F2, Ag5del_R1 and Ag5del_R2, and Ppd-D1_F1 and Ppd-D1_R1 (Bentley *et al.* 2011) to confirm the allelic composition at the *Ppd-A1* and *Ppd-D1* loci, respectively. Amplified PCR fragments were separated by electrophoresis in 2% agarose gel stained with Gel Green (Biotium, Hayward, CA, USA).

3.3.3 Transcription profile of Vrn-A1

Total RNA was extracted from the leaves of 4 weeks-old seedlings grown under long day (16 h) conditions without vernalization using TriPure isolation reagent (Roche, Mannheim, Germany), following the manufacturer instructions. RNA was treated with RNase-free DNase I (Takara, Ohtsu, Japan) to remove any genomic DNA. 1 µg RNA was used to synthesize first strand cDNA using Transcriptor first strand cDNA synthesis Kit (Roche). The first strand cDNA (50 ng) was used for the PCR using primers 5'-CCTGAACGGTATGAGCGCTAT-3' and 5'-GCATGAAGGAAGAAGATGAAG-3' for amplification of the *Vrn-1A* transcript (Diaz *et al*. 2012), and primers 5'-TCAACGAGGAATGCCTAG-TAAGC-3' and 5'-ACAAAGGGCAGGGACGTAGTC-3’ for the amplification of the ribosomal 18S gene as internal control gene (Fontecha *et al.* 2007). The PCR conditions were initial denaturation at 95°C for 5 min followed by 35

cycles at 94°C, 58°C and 72°C for 30 seconds each then final extension step at 72°C for 7 minutes.

3.3.4 Sequencing

The PCR products generated for the *Vrn-1A* were sequenced after cloning in pGEM-T easy vector (Promega, Madison, USA) using the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in an ABI 3130 genetic analyzer (Applied Biosystems). Sequences analysis was performed using version 10 of the GENETYX software (GENETYX Corporation, Tokyo, Japan).

3.3.5 Genetic analysis of the Vrn-A1

To investigate the genetic segregation of the early flowering in the introgression lines, a cross was made between CS and introgression line TAC1. The F_1 plants were self-pollinated to produce F_2 population. Phenotypic evaluation was carried out as described previously. Presence or absence of the alien chromosome was examined in all plants by specific markers for the alien chromosome (Table 3-2), chromosome counting and genomic *in situ* hybridization (GISH). Primer pair VRN1AF and VRN1-INT1R was used to examine the allelic constitution at the *Vrn-A1* locus.

3.3.6 Identification of the out crossing and point of the insertion

Six SSR primers (Table 3-3) located on the long arm of wheat chromosome 5 (5LA) were selected from the wheat SSR markers list deposited in USDA, GrainGenes (http://wheat.pw.usda.gov) to identify whether the alteration on *Vrn-A1* was due to outcrossing or not.

To identify the point of the alteration, early backcross generations of the production of the introgression lines and selfed seeds of early generation during the maintenance of the introgression lines were analyzed using the primer pair VRN1AF and VRN1-INT1R to identify the presence or absence of the insertion in *Vrn-A1* allele.

3.3.7 Statistical analysis

All data were subjected to analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test at $P<0.05$ using StatView software v. 5.0.1 (SAS Institute, Inc., USA).

3.4 Results

3.4.1 Flowering time

The previous results (Chapter 2) showed that under field conditions in Sudan (11 h day length), all introgression lines flowered earlier than CS, except TAC17, the latest flowering genotype, which was comparable to CS. TAC1 and TAC13, were the earliest-flowering lines. Under field conditions in Japan (13 h day length), the accessions mostly did not differ significantly in their flowering time, with the exception of TAC17, which was again the latest-flowering genotype (Fig. 3-1). TAC1 harbors homoeologous-group (HG) 2 chromosome of *L. racemosus*, whereas TAC13 harbors HG 5 chromosome (Larson *et al*. 2012). TAC17, in contrast, has HG 2 chromosome of *L. racemosus* in place of chromosome 2B of wheat (Qi *et al.* 1997).

TAC1, TAC13, and TAC17 were further compared with CS in a growth chamber under long-day conditions (16 h) without vernalization. All three lines flowered

significantly earlier than CS by an average of 34 days (Fig. 3-1). TAC13 flowered earlier than TAC1 by 9 days and TAC17 by 12 days.

3.4.2 Markers analysis and allelic composition at the Vrn and Ppd loci

Table 3-4 shows the allele combinations in the three introgression lines and CS. All the lines were tested with the primers VRN1AF and VRN1-INT1R for the *Vrn-A1* promoter region. CS showed a fragment larger than 700-bp, whereas all three introgression lines showed two PCR fragments larger than the expected fragment detected in CS (Fig. 3-2A). Yan *et al.* (2004a) found in TDD the presence of PCR products of two different lengths both of which larger than the fragment amplified in CS by insertion of 222-bp and 131-bp fold-back element; they designated this new allele *Vrn-A1a*. Based on the sequence of the TDD (GenBank AY616458) a new primer pair INS-7-F and INS-7-R (Table 3-1) was designed to target only the insertion of the fold-back elements identified in TDD. Using this primer pair the presence of the inserted fold-back element was confirmed in the introgression lines (Fig. 3-2B).

The fragment detected in CS (larger than 700-bp) is a characteristic of the dominant *Vrn-A1c* allele or the recessive *vrn-A1* allele (Yan *et al*. 2004a). To distinguish between these alleles, all lines were tested using the primer pair Intr1/A/F2 and Intr1/A/R3, and Intr1/C/F and Intr1/AB/R for the first intron of *Vrn-A1*. No PCR product was produced using the primer pair Intr1/C/F and Intr1/AB/R, whereas a 1068-bp fragment was produced in all lines using the primer pair Intr1/A/F2 and Intr1/A/R3, indicating that all the introgression lines lacked the large intron 1 deletion that defines the *Vrn-A1c* allele and that the 734-bp amplification product detected in CS is the recessive allele *vrn-A1c*.

Using the primer pair Intr1/B/F and Intr1/B/R3 for the dominant spring allele *Vrn-B1*, no PCR fragment was produced, whereas all the tested genotypes possessed a 1149-bp fragment using primers Intr1/B/F and Intr1/B/R4, indicating that these lines had the recessive allele *vrn-B1* (Fig. 3-2C). A 1671-bp fragment, the characteristic of the dominant *Vrn-D1* was generated in all genotypes using the primer pair Intr1/D/F and Intr1/D/R3, and no PCR product was obtained using the primer pair Intr1/D/F and Intr1/D/R4. This result indicates that all the tested genotypes had the dominant allele *Vrn-D1* (Fig. 3-2D). No allelic polymorphism was detected among the tested lines and CS in the *Vrn-3*, *Ppd-A1* and *Ppd-D1* loci except *Vrn-A1*.

In *L. racemosus*, no amplification was produced by any of the primer sets mentioned above, suggesting that its vernalization and photoperiod genes are different from those of wheat.

3.4.3 Expression analysis

The results of the markers analysis indicated that *Vrn-A1* allele of TAC1, TAC13 and TAC17 differ from CS by an insertion in the promoter region. To confirm the association between the detected difference in *Vrn-A1* and the observed early flowering, the expression of the *Vrn-A1* was investigated in the 4 weeks old seedlings grown without vernalization under long day condition (16 h). The results indicated that the *Vrn-A1* allele is highly expressed in TAC1, TAC13 and TAC17 compared to CS (Fig. 3-3).

3.4.4 Sequencing analysis

The sequencing analysis for the fragments generated for *Vrn-A1* form CS, TAC1, TAC13 and TAC17 using primer pairs VRN1AF and VRN1-INT1R, and INS 7-F and INS

7-R indicated that the promoters region of *Vrn-A1* of the three introgression lines was duplicated and differ from that of CS by an insertion of fold-back elements (Fig. 3-4). The sequences of the large and small fragments were similar to the sequences found in the spring wheat line TDD (Yan *et al*. 2004a) indicating that also the introgression lines had the 222-bp and 131-bp foldback element insertions in their promoters (Fig 3-2B, 3-4). As in TDD both fold-back elements were inserted in the same site and created the same 9-bp host direct duplication (HDD=TTAAAAACC) in the introgression lines (Figs. 3-6, 3-7). Outside the insertion, CS differed from the introgression lines and TDD by 2 SNPs (T/G at nucleotide 28 and A/G at nucleotide 889). TAC1 differed from CS, TDD and other introgression lines by 1 SNP (T/C) at nucleotide 134. On the other hand TAC13 showed an extra nucleotide (G) at nucleotide 508 (Fig. 3-5). In the large fold-back element (222-bp), TAC1 differed from TDD, TAC13 and TAC17 by one SNP at nucleotide 195 (T/G), whereas TAC17 differed by one SNP at nucleotide 199 (T/C) and insertion of one nucleotide (T) at position 171 (Fig. 3-6). In the small fold-back element (131-bp) the introgression lines differed from TDD by insertion of one nucleotide (C) at nucleotide 30 and deletion of one nucleotide (T) at position 143 (Fig. 3-7). As in TDD the small foldback element differ from the large fold-back element by one deletion of 91-bp started at position 73.

3.4.5 Genetic analysis of the F_2

All the F_1 plants produced from the cross of CS x TAC1 were monosomics having one alien chromosome. The F_1 plants flowered earlier than CS. Molecular analysis using primer pair VRN1AF and VRN1-INT1R indicated that all of them had the insertion of the

fold-back elements in their *Vrn-A1* promoter region. One plant was selected and self-pollinated to produce F_2 generation. The generated 37 F_2 plants were analyzed for the presence or absence of the alien chromosome and also the insertion of the fold-back elements in their promoters. The alien chromosome and the insertion segregated independently. Of the total number of plants, 22 (59.9%) had the alien chromosome, and 19 of the 22 plants (86.4%) carried the insertion of the fold-back element. In addition of the 15 plants without the alien chromosome, nine (60.0%) had the insertion. The F_2 plants segregated for the winter/spring allele at *Vrn-A1* in the ratio of 1 winter: 2 heterozygous: 1 spring ($\chi^2 = 1.8$, $P > 0.05$) indicating that the insertion is located in the wheat genome and confirmed the spring *Vrn-A1* allele.

3.4.6 Analysis of out crossing and point of the insertion

PCR analysis was performed using SSR markers for the long arm of wheat chromosome 5 (5LA), to identify whether the observed insertion in the introgression lines is due to outcrossing or not. DNA from TDD was used as control. Only marker *gwm126* showed polymorphism between CS and TDD. All the introgression lines had similar bands size to that amplified from CS using all SSR markers, indicating that this insertion might not be due to out crossing (Fig. 3-8A).

To identify the point of this insertion and explain the possible source of this inserted sequence, we used primer pair VRN1AF and VRN1-INT1R to detect the presence or absence of the insertion in 7 plants from BC_2F_2 and 2 plants from BC_2F_3. The number of chromosomes in the back cross lines ranged from 44 to 51. All the back cross lines showed

a fragment similar to the fragment of CS, indicating the absence of the insertion in these plants (Fig. 3-8B).

3.5 Discussion

The spring alleles of the *Vrn* genes are epistatic to the winter alleles (Pugsley, 1971), and the winter growth habit is observed only when all genes have recessive alleles. The *Vrn-A1a* allele is the most potent allele for promoting a spring growth habit, and provides complete elimination of vernalization, whereas *Vrn-B1* and *Vrn-D1* only partially eliminate the requirement (Pugsley, 1972). This confirms that CS has a vernalization requirement to promote flowering. The analysis of the allelic composition using specific markers indicated that CS had the recessive alleles *vrn-A1* and *vrn-B1* and the dominant allele *Vrn-D1* as expected, whereas the introgression lines TAC1, TAC13 and TAC17, possessed dominant allele *Vrn-A1* in addition to *vrn-B1* and *Vrn-D1*. Yan *et al.* (2004) characterized the dominant *Vrn-A1a* allele and showed that it differed from the recessive allele by insertion of 222-bp and 131-bp fold-back elements in its promoter region. Molecular analyses revealed that all of these addition lines also have duplicated insertions of the fold-back elements in *Vrn-A1* promoter regions as reported in *Vrn-A1a*.

In Sudan, the wheat season is short and warm, especially in central Sudan (18°N to 22°N), whereas the wheat season in Japan is longer and colder. In Japan, wheat crops benefit from a long and cold winter, with heading occurring at the end of winter and the beginning of spring. In Sudan, winter wheat (which requires vernalization for ear emergence) is not grown on account of the warm temperatures, whereas winter wheat can grow in Japan and reach its maximum yield potential. When the introgression lines were

evaluated in Japan, all of them except TAC17 flowered at close to the same time as their background, CS, but when they were evaluated in Sudan, TAC1 and TAC13 flowered significantly earlier and TAC17 flowered significantly later. In Japan, but not in Sudan, the wheat crop can achieve the vernalization requirement. Chinese Spring requires some vernalization to promote its flowering, as it only has the dominant allele *Vrn-D1*. This could explain the nearly flowering time in Japan and the late flowering of CS in Sudan where its vernalization requirement could not be achieved. On the other hand, the early flowering of TAC1 and TAC13 in Sudan and in the growth chamber, and the early flowering of TAC17 in the growth chamber, and the enhanced expression of *Vrn-A1* in the introgression lines compared to CS indicate that their vernalization requirement has been eliminated especially that they have the same photoperiod response alleles as CS (*Ppd-B1*). The early flowering of TAC1 and TAC13 can be attributed to the effect of the insertion of the fold-back elements at the *Vrn-A1* loci. Same insertion was detected in TAC17, but this line showed delayed flowering in Sudan and Japan under field conditions and early flowering in the growth chamber under long-day conditions. This reveals the absence of the photoperiod-response *Ppd-B1* allele on chromosome 2B, which is replaced in TAC17 by chromosome 2Lr#1 of *L. racemosus.*

These results conclude that the early flowering observed in the two addition lines and the substitution line results from eliminating the vernalization requirement by the presence of fold-back elements insertion at *Vrn-A1* loci, and consequently the presence of *Vrn-A1a* allele.

The segregation of the spring/winter alleles in F_2 population in the mendilian ratio 1:2:1 indicates that the insertion is in the wheat genome and the early flowering is not due to the effect of the alien chromosome. Also the presence of large fragment and small fragment of the fold-back elements in all F_2 progenies possessed the spring growth habit confirm that those fragments were linked as reported by Yan *et al.* (2004a).

We could not generate any PCR product from *L. racemosus* using primer pairs VRN1AF and VRN1-INT1R, and INS-7-F and INS-7-R for the *Vrn* allele. Thus, the source of the insertion in the introgression lines could be due to activation of the mobile element stimulated by the presence of the alien chromosome or to outcrossing or contamination during production or maintenance of the introgression lines, though the latter possibility is very less because the materials were carefully produced and the basic morphology of the introgression lines are similar to the recipient cultivar, CS. To deny the possibility of outcrossing or contamination of the materials, we conducted further molecular analysis using SSR markers. The results reduced the possibility of outcrossing; especially that wheat is highly self-pollinated crop with outcrossing rate usually less than 1% (Hucl 1996). In addition, no morphological change and genetic segregation was observed in these lines during 3 generations of seed maintenance and multiplication. However, differences (SNPs) were observed between CS and the introgression lines in the promoter of *Vrn-A1* (Fig 5). The observed differences in the promoter sequences between the introgression lines suggest that those insertions are independent events, especially that those lines were developed independently; TAC1 was produced independently in Japan (Kishii *et al*. 2004) whereas TAC13 and TAC17 produced in China (Qi *et al*. 1997). However, the structure of the

insertion is basically same (two insertions and special structure). This fact clearly indicates that this insertion occurred inevitably but accidentally by unknown genetic mechanism. The alien chromosomes of TAC1 and TAC17 belong to homoeologous group 2 but these lines are produced independently in Japan and China. The alien chromosome of TAC13 belongs to homoeologous group 5. These suggest that a specific alien chromosome was not the trigger of this rearrangement.

In plants including wheat, genomic reorganizations and modifications has been reported. These modifications include structural rearrangements in chromosomes (Leitch and Bennett 1997) and sequences (Song *et al.* 1995, Wendel *et al.* 1995), changes of gene expression (Comai *et al.* 2000, Kashkush *et al.* 2002) and activation of transposons (Kashkush *et al.* 2002, 2003). The introgression lines used in this study had a pair of alien chromosomes added to the wheat genome, therefore it might be expected that some genetic changes taken place during the production and maintenance of these lines, especially that Fu *et al*. (2013) illustrated that monosomic wheat-rye addition lines could induce different and drastic genetic/epigenetic variations and these variations might not be caused by introgression of rye chromatins into wheat.

Yan *et al.* (2004a) reported that the number of the fold-back elements in the genome of the tetraploid wheat is approximately 375 copies. This suggests the abundance of this fold-back element in wheat genome. Based on these results it might be possible to propose that this insertion is likely due to genetic event occurred during maintenance of these introgression lines rather than outcross pollination. However, more research is needed to

clarify this proposal and detect the point of the insertion by analyzing different generation of maintenance of these lines.

Most of the wild *Triticeae* species have a winter growth habit, suggesting that the recessive *vrn-1* allele is the ancestral feature (Goncharov 1998). Golovnina *et al.* (2010) studied the molecular variability of the wheat *Vrn-1* promoter region in diploid, tetraploid and hexaploid wheat accessions to clarify the complicated molecular basis of spring vs. winter growth habit; they concluded that DNA transposon insertions first occurred in polyploid species. At the same time, the duplication of the promoter region was observed in A genomes of polyploid species. Yan *et al.* (2004a) reported that the duplication of the promoter and the insertion of the fold-back element are found only in hexaploid wheat. On the basis of our results, we speculate that the insertion of the fold-back element and the duplication of the promoter in spring wheat are due to polyploidization and occurred after the addition of the diploid DD genome to the tetraploid AABB genome.

We conclude that the early flowering of the introgression lines is due to an insertion in the promoter of the *Vrn-A1* allele occurred during the maintenance of the introgression lines; this insertion might result from activation of a mobile element in the wheat genome caused by the presence of an alien chromosome. These results suggest that the evolution of spring wheat from winter wheat through promoter duplication occurred after the polyploidization due to the addition of the DD genome to the AABB genome.

Table 3-1. List of the primers used to detect the allelic composition at the *Vrn* and *Ppd* genes in CS and the introgression lines TAC1, TAC13 and TAC17

Primer position	Marker name	Sequence	Reference
Vrn-A1 Promoter	VRN1AF	GAAAGGAAAAATTCTGCTCG	Yan *et al*. 2004
	VRN1-INT1R	GCAGGAAATCGAAATCGAAG	Yan *et al.* 2004
A, B, D genome	VRN1AF	GAAAGGAAAAATTCTGCTCG	Yan *et al.* 2004
specific primers	VRN1BF	CAGTACCCCTGCTACCAGTG	Yan *et al.* 2004
	VRN1DF	CGACCCGGGCGGCACGAGTG	Yan *et al.* 2004
	VRN-1R-A	TGCACCTTCCCCCGCCCCAT	Yan *et al*. 2004
	VRN-1R -B,D	TGCACCTTCCCGCGCCCCAT	Yan *et al.* 2004
VRN-A1	INS-7-F	GCCAGATCCCTTTAAAAACCG	
	INS-7-R	CCAGGCCAAAACGAGGATTC	
VRN-A1	Intr1/A/F2	AGCCTCCACGGTTTGAAAGTAA	Fu *et al*. 2005
	Intr1/A/R3	AAGTAAGACAACACGAATGTGAGA	Fu *et al.* 2005
vrn-A1	Intr1/C/F	GCACTCCTAACCCACTAACC	Fu *et al.* 2005
	Intr1/AB/R	TCATCCATCATCAAGGCAAA	Fu *et al.* 2005
VRN-B1	Intr1/B/F	CAAGTGGAACGGTTAGGACA	Fu *et al.* 2005
	Intr1/B/R3	CTCATGCCAAAAATTGAAGATGA	Fu *et al.* 2005
vrn-B1	Intr1/B/F	CAAGTGGAACGGTTAGGACA	Fu *et al.* 2005
	Intr1/B/R4	CAAATGAAAAGGAATGAGAGCA	Fu *et al.* 2005
VRN-D1	Intr1/D/F	GTTGTCTGCCTCATCAAATCC	Fu *et al.* 2005
	Intr1/D/R3	GGTCACTGGTGGTCTGTGC	Fu *et al.* 2005
vrn-D1	Intr1/D/F	GTTGTCTGCCTCATCAAATCC	Fu *et al.* 2005
	Intr1/D/R4	AAATGAAAAGGAACGAGAGCG	Fu *et al.* 2005
VRN3	FT-B-INS-F	CATAATGCCAAGCCGGTGAGTAC	Yan *et al.* 2006
	FT-B-INS-R	ATGTCTGCCAATTAGCTAGC	Yan *et al.* 2006
	FT-B-NOINS-F	ATGCTTTCGCTTGCCATCC	Yan *et al*. 2006
	FT-B-NOINS-F2	GCTGTGTGATCTTGCTCTCC	Yan *et al.* 2006
	FT-B-NOINS-R	CTATCCCTACCGGCCATTAG	Yan *et al.* 2006
Ppd-A1	Ag5del_F2	TGTCACCCATGCACTCTGTTT	Bentely *et al.*2011
	Ag5del_R1	GAGCAAGGGATTGAGACTGC	Bentely *et al.*2011
	Ag5-del_R2	CTGGCTCCAAGAGGAAACAC	Bentely *et al.*2011
Ppd-D1	Ppd-D1_F	ACGCCTCCCACTACACTG	Bentely *et al.*2011
	Ppd-D1_R1	TGTTGGTTCAAACAGAGAGC	Bentely *et al.*2011

Table 3-2. List of *Leymus* SSR markers used to test the presence or absence of the alien chromosome

Leymus SSR	Forward primer	Reverse primer
Ltc0090	AACTAAGCACACGATGGGAGATG	TACTATACGTGCGCAGAGCAACAT
Ltc0543	GAGGGAAAGATGAGGGAGCTG	GTTGACGTCCTCCCACCATC
Ltc0682	TTTTTGTATCGCAAGTCACTAAGGT	AATTAGTTGTTGAGCGTCTTGCAT
Ltc0697	CAGGTTCTGGAAGAACAAGAGGAA	TACTCCTGAACCGAGAGCAAAGAC
Ltc0761	GCCTGGAGACTCTACTTGCTGTTT	TTTTTAAAGCAAGCCACGAGGTAA
Ltc1142	AAACCCTACCCCTCGAGCAAC	GTCATCGATCTCGACCTTCTTCTG
Ltc1148	ACGGCAACCAGTACCACGTC	GAGGGTTCGCTGTCATCGTC

Table 3-3. List of the long arm of chromosome 5 (5LA) SSR markers used to test the possibility of outcrossing at chromosome 5LA

Marker name	Sequence (F)	Sequence (R)
gwm126	CACACGCTCCACCATGAC	GTTGAGTTGATGCGGGAGG
gwm156	CCAACCGTGCTATTAGTCATTC	CAATGCAGGCCCTCCTAAC
gwm291	CATCCCTACGCCACTCTGC	AATGGTATCTATTCCGACCCG
wmc110	GCAGATGAGTTGAGTTGGATTG	GTACTTGGAAACTGTGTTTGGG
wmc524	TAGTCCACCGGACGGAAAGTAT	GTACCACCGATTGATGCTTGAG
cfa2155	TTTGTTACAACCCAGGGGG	TTGTGTGGCGAAAGAAACAG

Table 3-4. Allele combination of *Vrn* and *Ppd* genes of CS and introgression lines TAC1, TAC13 and TAC17

Genotypes	*Vrn-A1*	*Vrn-B1*	*Vrn-D1*	*Vrn-B3*	*Ppd-A1*	*Ppd-B1*	*Ppd-D1*
Chinese Spring	*vrn-A1*	*vrn-B1*	*Vrn-D1*	*vrn-3*	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*
TAC1	*Vrn-A1a*	*vrn-B1*	*Vrn-D1*	*vrn-3*	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*
TAC13	*Vrn-A1a*	*vrn-B1*	*Vrn-D1*	*vrn-3*	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*
TAC17	*Vrn-A1a*	*vrn-B1*	*Vrn-D1*	*vrn-3*	*Ppd-A1b*	*Ppd-B1a*	*Ppd-D1b*

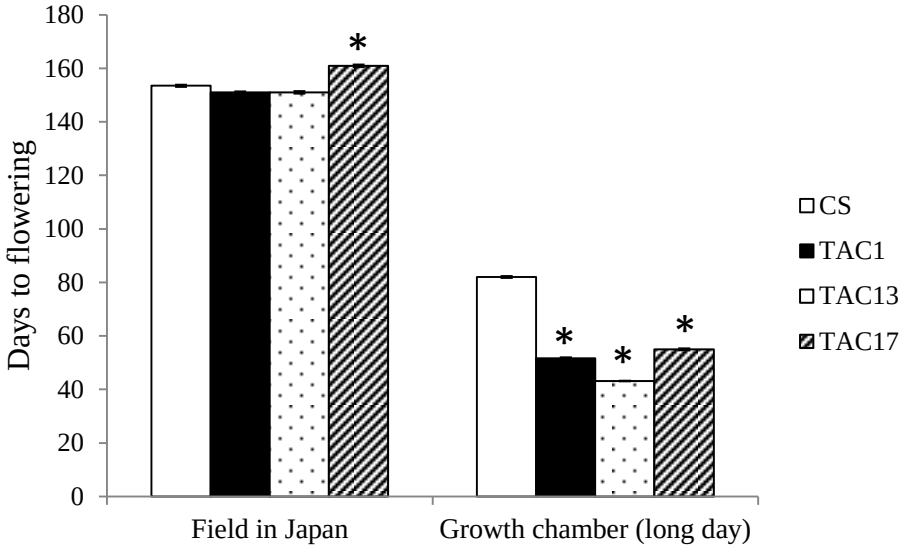


Fig. 3-1. Days to flowering of CS and introgression lines (TAC1, TAC13 and TAC17) evaluated in field in Japan and in growth chamber under long (16 h) conditions without vernalization Astrics indicate the significant difference from CS (P <0.05, Fisher's PLSD test).

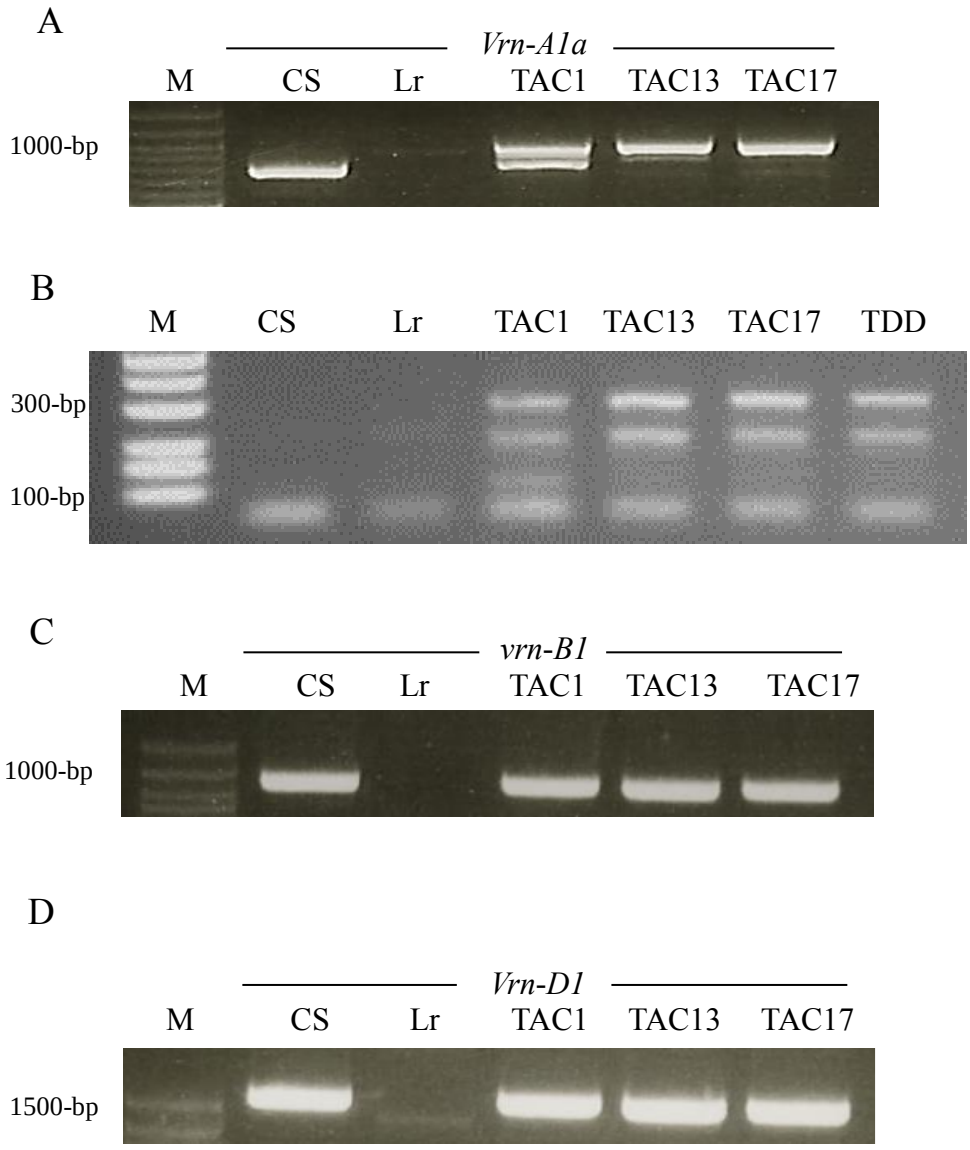


Fig. 3-2. PCR amplification to detect the allelic composition at *Vrn-A1, Vrn-B1* and *Vrn-D1* loci using (A) primer pair VRN1AF and VRN1-INT1R for *Vrn-A1*alleles, (B) INS-7-F and INS-7-R to detect the fold-back elements insertion, (C) to detect *Vrn-B1* alleles and (D) to detect *Vrn-D1* alleles. Lr, *Leymus racemosus*. TDD, Triple Dirk D.

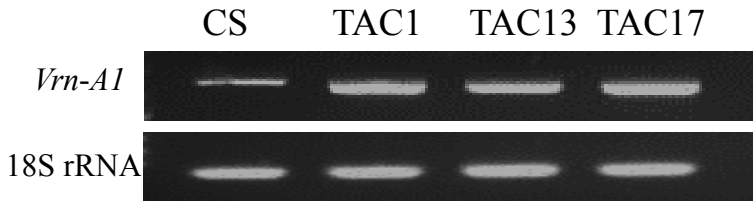


Fig. 3-3. RT-PCR analysis for the expression of the *Vrn-A1*. RNA extracted form 4 weeks-old seedling growth under long day conditions (16 h) without vernalization.

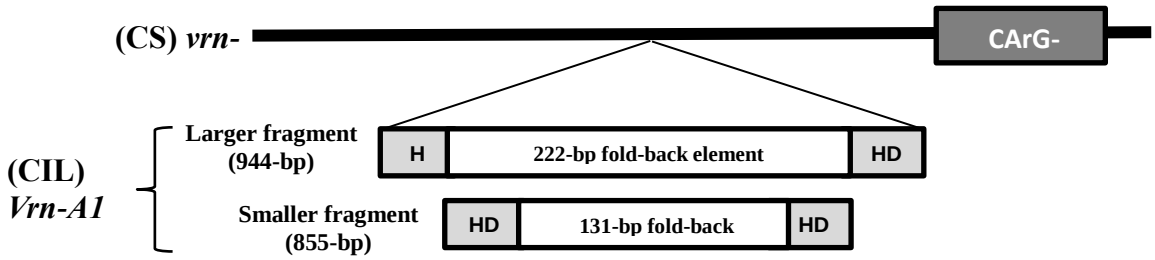


Fig. 3-4. Schematic diagram illustrate the difference in the promoter region of CS (*vrn-A1*) and chromosome introgression lines (CIL) (*Vrn-A1*) duplicated promoter with the insertion of the large fold-back element (222-bp) and small fold-back element (131-bp). Host Direct duplication (HDD) region is indicated in light gray, and the putative CArG- box region is indicated in dark gray.

Fig. 3-5. Section of the sequence alignment showing the differences between CS, introgression lines (TAC1, TAC13 and TAC17) and TDD in the promoter of the *Vrn-A1* amplified by primer pair VRN1AF and VRN1-INT1R.

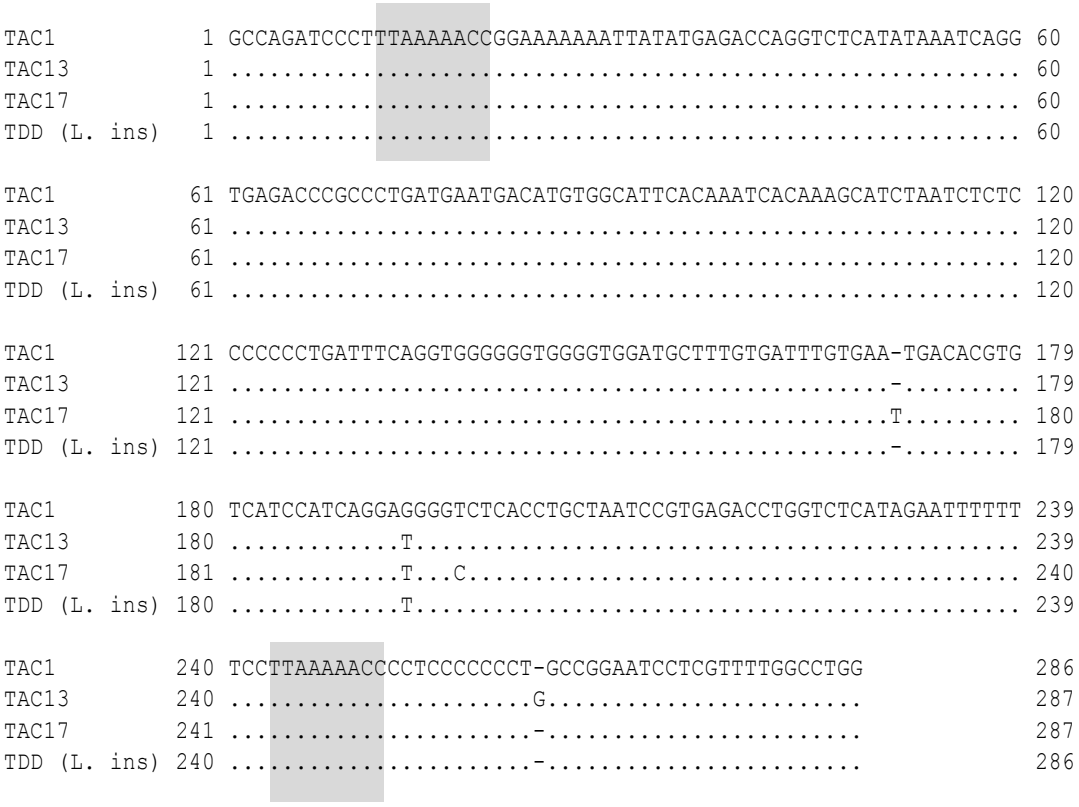


Fig. 3-6. Sequence alignment of the large insertion (222-bp) in the promoter of TAC1, TAC13, TAC17 and TDD amplified by primer pair INS-7-F and INS-7-R. The host direct duplication (HDD) is highlighted.

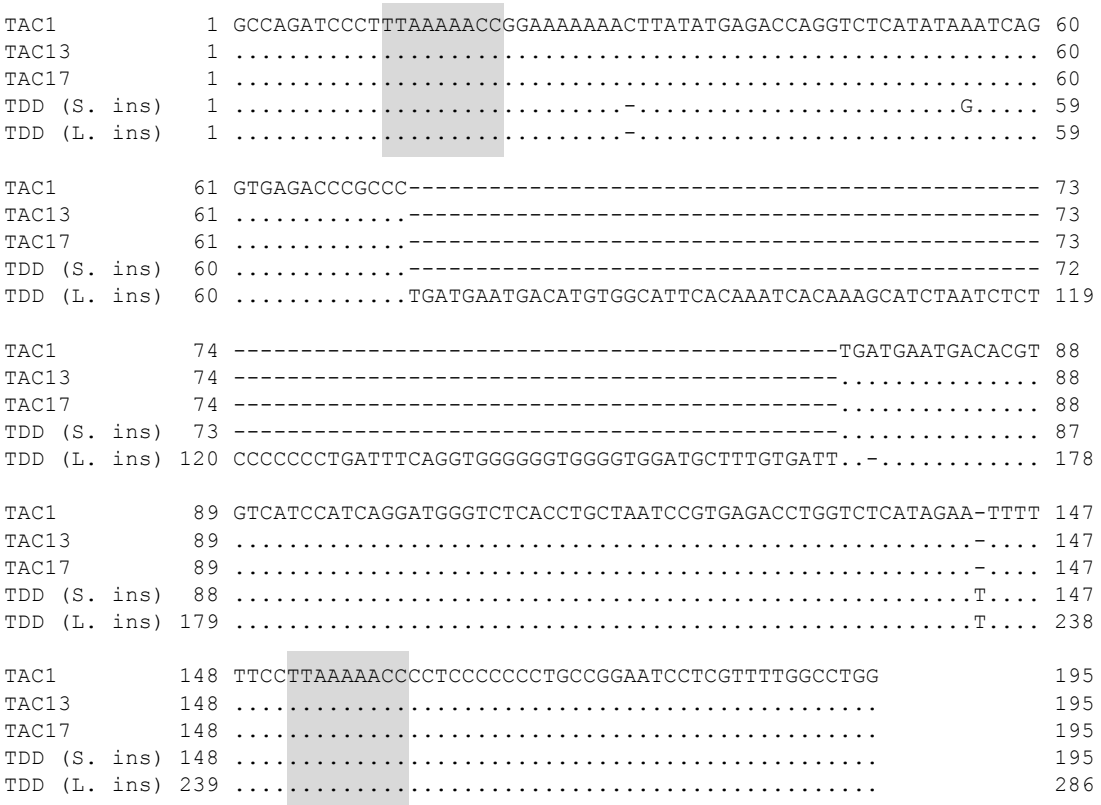


Fig. 3-7. Sequence alignment of the small insertion (131-bp) in the promoter of TAC1, TAC13, TAC17 and TDD amplified by primer pair INS-7-F and INS-7-R. The host direct duplication (HDD) is highlighted. The 91-bp deletion is indicated by dashes.

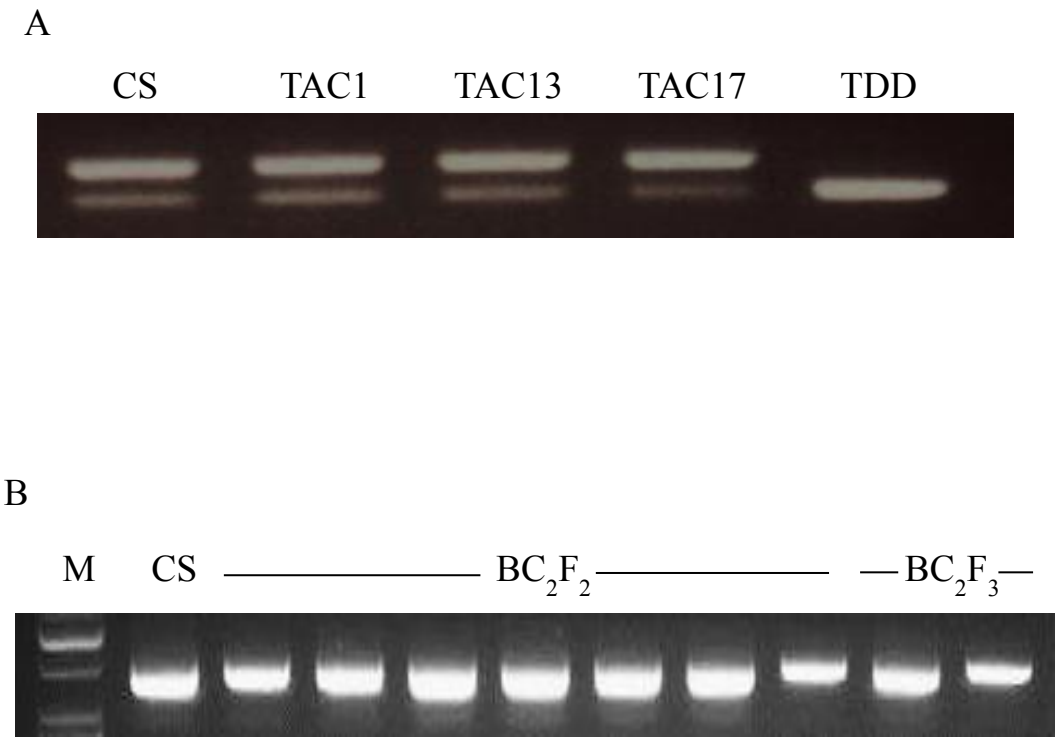


Fig. 3-8. PCR analysis using (A) SSR marker *gwm 126* of the long arm of chromosome 5LA to test the possibility of the outcrossing. (B) specific *Vrn-A1a* marker to detect the presence of the inserted promoter in early introgression lines backcross generations.

General discussion and conclusion

Genetic erosion caused by modem cultivation procedures has narrowed the genetic base of many crops, including common wheat. Wild relatives of wheat are an important source to enrich the wheat genetic diversity. In this study the results indicated clearly that the chromosomes of *L. racemosus* enhanced the wheat aluminum tolerance and heat stress tolerance. In addition, the alteration of the flowering time observed in the introgression lines result from genetic change probably due to the presence of the alien chromosome. This indicates that not only the alien chromosome is useful for the wheat breeding but also the genetic changes that may result from the presence of the alien chromosome in the wheat background could provide new source of genetic diversity needed for wheat improvement. Moreover, this study revealed the importance of the chromosome addition and substitution lines as potential genetic materials for identification and discovery of new genes, especially when the phenotypic and genotypic evaluation of the wild relative has some limitations.

These introgression lines identified in this study are useful for wheat improvement for aluminum and heat stress tolerance, however they cannot be used directly in breeding programs. These genetic stocks could therefore be used as a bridge to introduce the valuable *Leymus* traits identified in this study into superior wheat backgrounds, thus enhancing wheat adaptation and maximizing yield potential under stressed environments.

Summary

The world population is growing exponentially and there is an urgent need to increase and stabilize the world food production. Wheat is the most important food crop worldwide, and thus the food sufficiency could be achieved through development of new stress tolerant and well adapted wheat cultivars with high yield potential. For efficient and successful breeding of outstanding and well adapted cultivars genetic diversity is needed. However, since the available genetic diversity for wheat breeding is very narrow, this study was undertaken to explore the potentiality of genes from *Leymus racemosus*, a wheat wild relative for wheat breeding through physiological and molecular evaluation of wheat - *L. racemosus* introgression lines for aluminum and heat stress tolerance.

Chapter 1 describes the impact of *L. racemosus* chromosomes on wheat tolerance to aluminum (Al) toxicity. The introgression lines and their wheat background Chinese Spring (CS) were evaluated in hydroponics system under various concentration of Al to identify new genetic resources to improve wheat tolerance to Al, and to identify the chromosomes harboring the tolerance genes. Al uptake, oxidative stress, and cell membrane integrity were also investigated. The results indicated that *L. racemosus* chromosomes A and E incorporated in TAC1 and TAC3, respectively, significantly enhanced the Al tolerance of wheat in term of relative root growth. At the highest Al concentration tested (200 µM), TAC3 had the greatest tolerance. The introgressed chromosomes did not affect Al uptake of the tolerant lines. The improved tolerance conferred by chromosome E was attributing to improved cell membrane integrity.

Chapter 2 describes the impact of *L. racemosus* chromosomes on wheat tolerance and adaptation to heat stress. Wheat - *L. racemosus* chromosome introgression lines and their parent CS were evaluated in a growth chamber at the seedling stage and in the field at the reproductive stage in two heat-stressed environments in Sudan. Optimum and late planting were used to ensure exposure of the plants to heat stress at the reproductive stage. The results revealed the impact of several *Leymus* chromosomes in improving wheat adaptation and tolerance to heat: Three lines possessed enhanced adaptation, whereas two showed high heat tolerance. Two addition lines showed a large number of kernels per spike, while one possessed high yield potential. The findings suggest that these genetic stocks could be used as a bridge to introduce the valuable *Leymus* traits into a superior wheat genetic background, thus helping maximize wheat yield in heat-stressed environments.

Chapter 3 describes the genetic basis of the early heading and maturity observed in three introgression (TAC1, TAC13 and TAC17) lines under field conditions in Sudan. It was first hypothesized that the vernalization requirement of CS was cancelled in the introgression lines, and this cancellation led to the early flowering. Specific molecular marker analysis revealed that these lines had the dominant *Vrn-A1* allele, whereas CS had the winter recessive *vrn-A1* allele. Unlike the CS winter allele, in the promoter region, the spring *Vrn-A1* allele of all the introgression lines had a large insertion of 220-bp and a small insertion of 131-bp. Sequence analysis indicated that the large insertions of the introgression lines were similar to the insertion of the spring genotype Triple Dirk D (TDD). The insertion of TAC1 showed one SNP (T/G) to CS, whereas TAC17 showed an extra nucleotide (T) in addition to one SNP (T/C). The small insertions of the introgression lines

differed from that of TDD by insertion, deletion and inversion events. Results of molecular marker analysis exclude the possibility of unexpectedly occurred outcrossing and suggested that this insertion is due to genetic events occurred during the maintenance of the introgression lines.

This study indicated clearly the potentiality of utilization the traits and genes of wheat wild relative *L. racemosus* to improve wheat tolerance to aluminum and heat stresses. Moreover, this study revealed the importance of the chromosome addition and substitution lines as potential genetic materials for identification and discovery of new genes, especially when the phenotypic and genotypic evaluation of the wild relative has some limitations.

Summary (in Japanese)

世界人口は急激に増加し、世界の食糧生産を増加させ安定化させる緊急な必要性がある。コムギは世界で最も重要な食糧作物であり、したがって、食糧の需要は、新規の高生産性かつストレス耐性・高適応性コムギ品種を開発することにより実現できるであろう。傑出し適応的な品種を効果的に確実に行うには、遺伝的多様性が必要である。しかし、コムギの育種に利用可能な遺伝的多様性は非常に狭い。本研究は、コムギ近縁植物、オオハマニンニク染色体導入コムギ系統のアルミニウムと高温耐性の生理学的、分子的評価を通じ、オオハマニンニクの遺伝子がコムギ育種への可能性を明らかにするために企画した。

第 1 章ではアルミニウムの毒性に対するコムギの耐性に関するオオハマニンニクの染色体の影響について記述した。コムギのアルミニウム耐性を改善するための遺伝資源を同定するために、導入系統とコムギの遺伝的背景である Chinese Spring (CS)を水耕栽培システムで評価し、耐性遺伝子を保有する染色体を同定した。アルミニウムの取り込み、酸化ストレス、細胞膜完全性も合わせて調査した。その結果、それぞれ系統 TAC1 と TAC3 に存在するオオハマニンニクの染色体 A および E が、根の成長において、有意にコムギのアルミニウム耐性を向上させることを見いだした。検査した中で最も高いアルミニウム濃度(200 μM)では、TAC3 が最高の耐性を示した。導入された染色体はアルミニウムの取り込みに影響はしなかった。染色体 E によって付与される耐性は細胞膜の完全性に起因していた。

第 2 章では、オオハマニンニク染色体がコムギの高温耐性および適応性に関する影響について記述した。ハマニンニク染色体を導入されたコムギの系統と親である Chinese Spring の苗を人工気象機で、また生殖期にスーダンの 2 つの環境下で圃場調査を行った。生殖期においては、適性および遅延播種により高温ストレスを確実に曝した。その結果、コムギの高温適応と耐性を改善するいくつかのハマニンニク染色体の影響を明らかにすることができた。つまり、3 系統は適応性を

もち、また 2 系統は高温耐性を示した。2 つの添加系統は穂あたり粒数を増加させ、１系統は高い収量ポテンシャルを示した。これらの知見から、これらの遺伝的系統は有用なオオハマニンニクの性質を、優れたコムギの遺伝的背景に導入するときの橋渡しとして利用し、高温ストレス環境でコムギの生産を最高にするために寄与することを示している。

第3章では、３つの導入系統(TAC1, TAC13 and TAC17)のスーダンの条件における早生および早熟性の遺伝的基礎について解説する。まず、導入系統においてCSの春化要求性が解除され、これにより出穂が早まるとの仮説を立てた。特定の分子マーカー分析により、CSは秋播性の対立遺伝子*vrn-A1*をもつのに対し、これらの系統は優性の対立遺伝子*Vrn-A1*をもつことを示した。CSの秋播性遺伝子とは異なり、導入系統すべての春播性遺伝子*Vrn-A1*遺伝子は、そのプロモーター領域に220-bpの大きい挿入と131-bpの小さい挿入をもっていた。配列解読により、導入系統の大きい挿入は、春播遺伝子型をもつTriple Dirk D (TDD)と類似の挿入をもつことがわかった。TAC1の挿入はCSに対し、1つのSNP(T/G)をもち、TAC17は2つの過剰塩基(T)と1つのSNP(T/C)をもっていた。小さい挿入配列はTDDの配列とは、挿入、欠失および逆位によって異なっていた。分子マーカーの結果から、予期せず起こる他家受精の可能性のないことが分ったため、この挿入は導入系統の維持の過程で起こった遺伝的な事象であることが示唆された。

この研究はコムギの近縁野生植物、オオハマニンニクの形質と遺伝子が、コムギのアルミニウムおよび高温への耐性を改良するために利用できる事を示した。さらに、この研究は、特に野生植物の表現型および遺伝子型の評価に限界があるとき、染色体添加や置換系統が新規遺伝子の同定と発見のための優れた遺伝的素材であることを明らかにした。

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List of published papers

Chapter1:

Title: Enhancement of aluminum tolerance in wheat by addition of chromosomes from the wild relative *Leymus racemosus*

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