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

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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. We used 30 wheat alien chromosome addition lines to identify new genetic resources to improve wheat tolerance to Al and to identify the chromosomes harboring the tolerance genes. We evaluated these lines and their wheat background Chinese Spring for Al tolerance in hydroponic culture at various Al concentrations. We also investigated Al uptake, oxidative stress and cell membrane integrity. 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. We attribute the improved tolerance conferred by chromosome E to improved cell membrane integrity. Chromosome engineering with these two lines could produce Al-tolerant wheat cultivars.

Key Words: aluminum tolerance, *Leymus racemosus*, addition line, wheat.

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 (Kochian *et al.* 2004, Ma *et al.* 2001). 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, 1997b, 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).

Leymus is a genomically defined, allopolyploid, Triticeae genus ($2n = 28$, 56 genome NsNsXmXm, NsNsNsNsXmXmXmXm, respectively) with 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

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Table 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	NA[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	6	44	Q	Qi *et al.* 1997
TACBOW0015	*L. racemosus* 7Lr#1 addition	3, 7	44	R	Qi *et al.* 1997
TACBOW0016	*L. racemosus* ?Lr#1 addition	NA	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] NA, not available.

areas, as well as shady and 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). Several *Leymus* species including *L. racemosus* have been hybridized successfully 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). The importance of *Leymus* species generally and *L. racemosus* particularly as novel sources for many economically important traits led us 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.

Materials and Methods

Plant materials and growing conditions

We studied 13 wheat-*L. racemosus* addition and 2 wheat-*L. racemosus* substitution lines (Table 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 glass house 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.

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.

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.

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).

RT- PCR analysis

Total RNA was extracted from the roots of Al treated and non-treated seedlings after 48 h of 25 μM 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′-CGTGAAAGCAGCGGAAAGCC-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 (Supplemental Table 1).

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 60°C for 3days then SDW and RWD were measured. For evaluation of Al tolerance we adopted the 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: Integrated score = absolute value of (SPAD value × 0.2 + tillers/plant × 0.2 + shoot dry weight × 0.2 + root dry weight × 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).

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).

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 $100 \times [1 - (EC1/EC2)]$.

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.

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).

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).

Results

Effect of aluminum on root growth

To identify the best concentration to screen the addition lines, we 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. 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. 1B). Addition lines A, E and O had 94, 77 and 79% RRG, respectively, compared to only 57% in CS (Fig. 1B, 1C). On the other hand, the RRG of lines H, N, R and S was significantly lower than that of CS (Fig. 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. 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), we 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

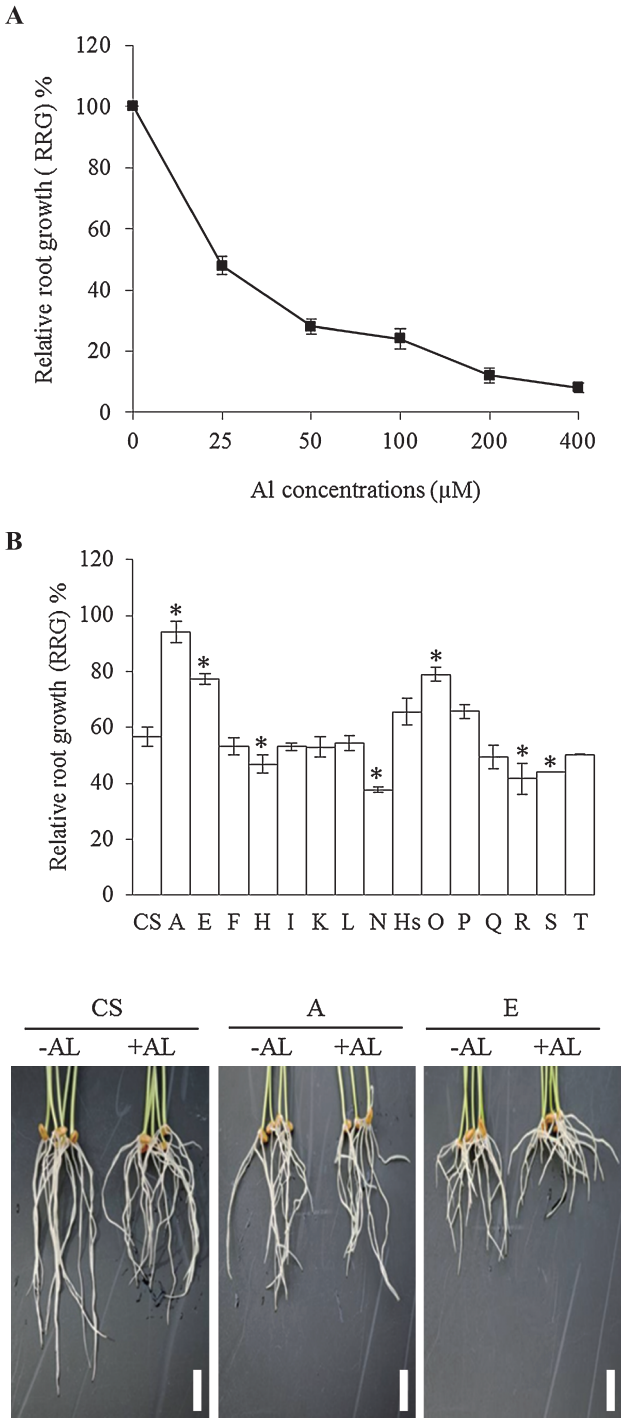

Fig. 1. Effect of Al on root growth of CS and *Leymus racemosus* chromosome addition lines. (A) The effect of different concentration of Al in relative root growth (RRG) of CS. Seedlings were exposed to 0, 25, 50, 100, 200, 400 µM Al for 48 h. Values are the mean of 10 replicated seedlings and the vertical 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.

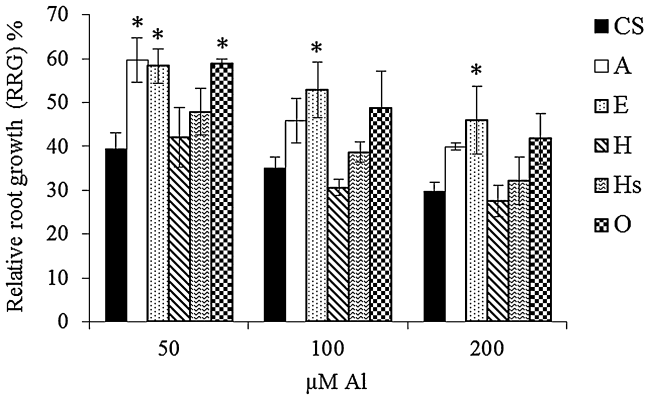

Fig. 2. Dose response evaluation of relative root growth (RRG) of CS and selected addition lines. Seedlings were exposed to 50, 100, or 200 μM Al for 48 h. Values are means ± SEM ($n = 2$); each replicate included 12 seedlings. Asterisks indicate significant differences from CS ($P < 0.05$, Fisher's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

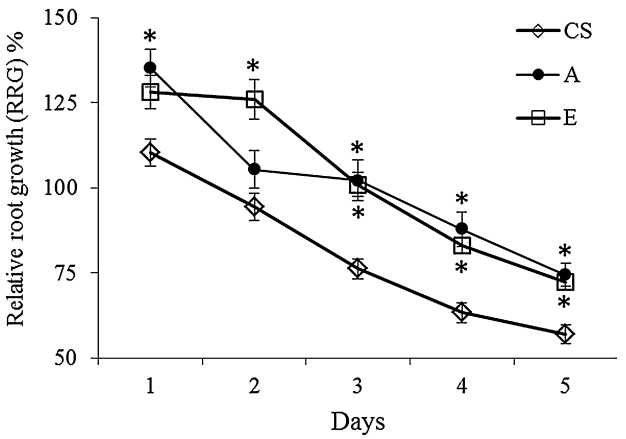

Fig. 3. Time course of effects of Al on CS and addition lines A and E. Seedlings were exposed to 10 μM Al for 5 days and the relative root growths were measured daily. Values are means ± SEM ($n = 15$) of the relative root growth (RRG) for each 24-h period. $P < 0.05$, Fisher's PLSD test.

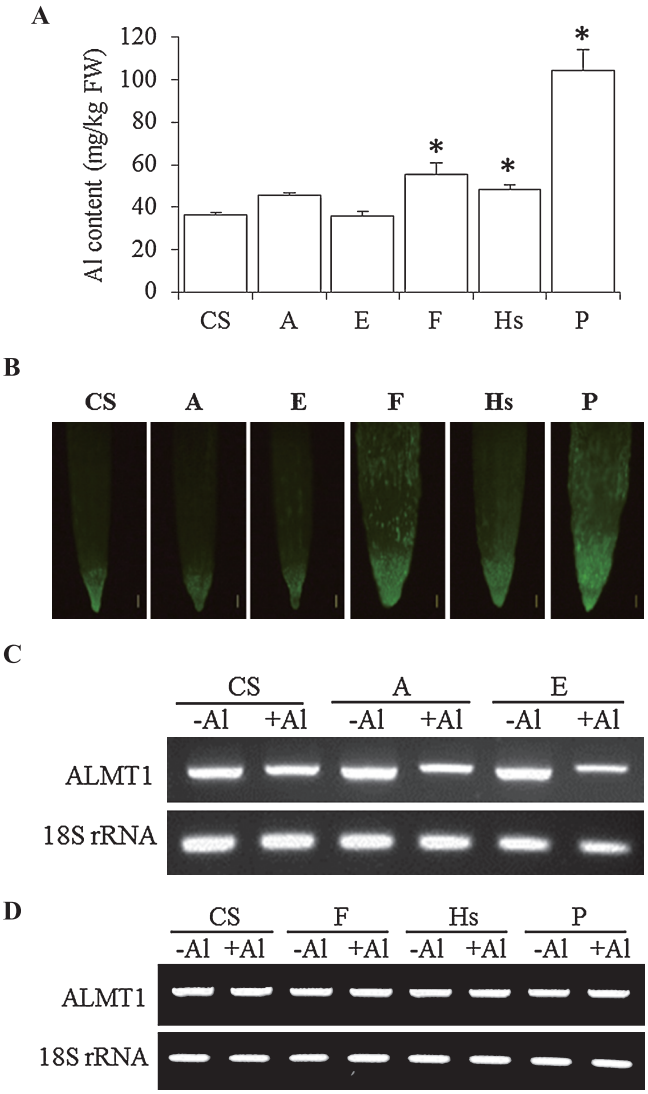

Fig. 4. Al accumulation and *ALMT1* expression in the roots. (A) Al content of CS and selected addition lines exposed to 25 μM Al for 48 h. Values are means ± SEM ($n = 3$). Asterisks indicate significant differences from CS ($P < 0.05$, Fisher's PLSD test). (B) Al localization in root tips of CS, A, 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.

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. 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, we tested the 15 HG 2 chromosome addition lines at 25 μM 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 (data not shown).

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, we determined the Al contents 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. 4A). In contrast, addition lines A and

Table 2. 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 ($P < 0.05$).

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. 4B).

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. 4C). In contrast, lines F, Hs and P showed the same level of expression of CS (Fig. 4D). 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 (data not shown).

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 2). 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 2). 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.

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

We 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. 5A). Treatment for 48 h reduced the integrity in the three lines

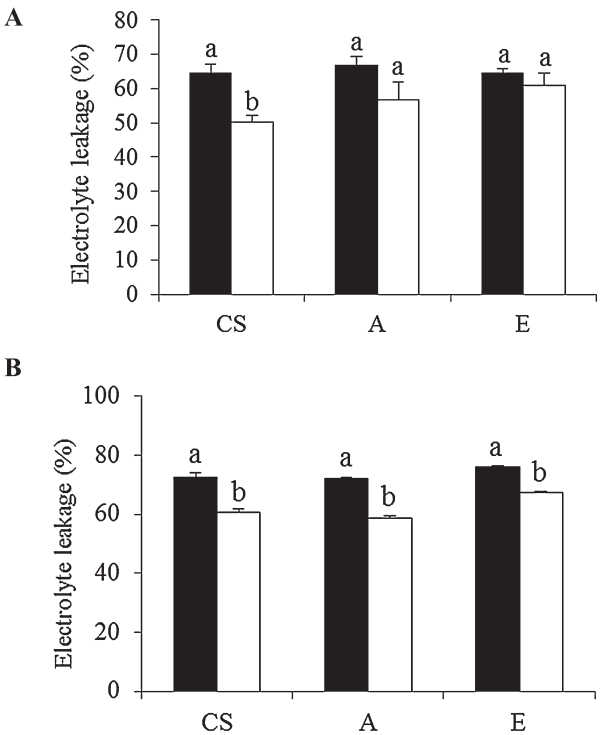

Fig. 5. Electrolyte leakage, indicator of the loss of plasma membrane integrity, of the roots of CS, A and E after (A) 24 h and (B) 48 h at 0 (dark bars) or 25 μM Al (white bars). Values are means ± SEM ($n = 4$). Each replication included 4 replicated seedlings. Means with different letters differ significantly ($P < 0.05$, Fisher's PLSD test).

significantly, by 35% to 47% (Fig. 5B). 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. 6A). 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. 6B).

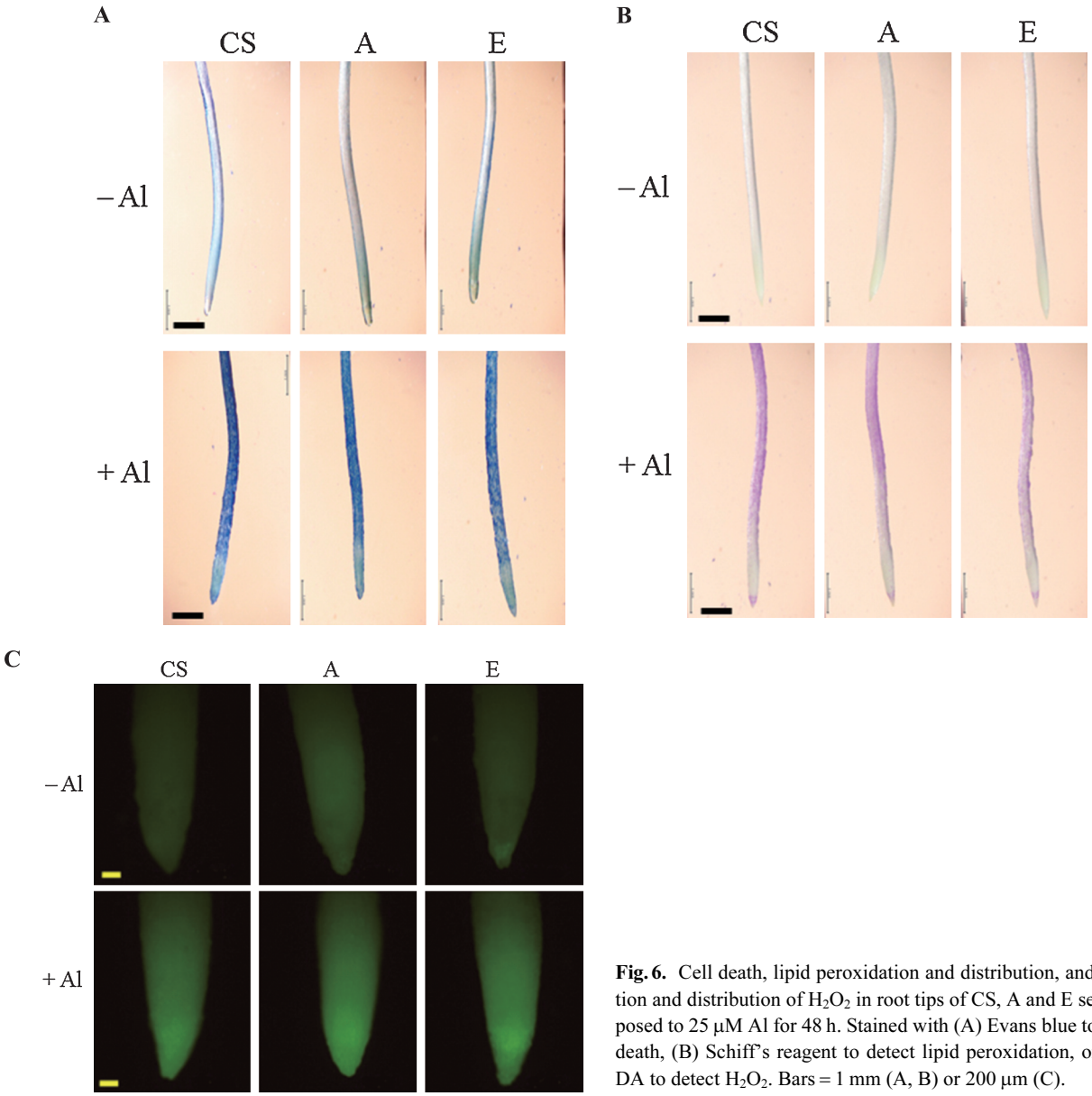

Fig. 6. Cell death, lipid peroxidation and distribution, and accumulation and distribution of H_2O_2 in root tips of CS, A and E seedlings exposed to 25 μM Al for 48 h. Stained with (A) Evans blue to detect cell death, (B) Schiff's reagent to detect lipid peroxidation, or (C) DCF-DA to detect H_2O_2. Bars = 1 mm (A, B) or 200 μm (C).

DCF-DA staining indicates that line A slightly accumulated more H_2O_2 than CS and line E when grown without Al (Fig. 6C). 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.

Discussion

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 (Fig. 1B, 2), while at higher concentrations tested, E performed better (Fig. 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. 3). Therefore, we conclude that this result is 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). We firstly 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, our 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. 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 (Kishii *et al.* 2004, Qi *et al.* 1997), we 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. We screened 15 addition lines from 11 species all of which harbor HG 2 chromosomes, but we did not find any tolerant lines. 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.

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. 4A, 4B and Table 2). 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 2). 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 (Raman *et al.* 2005, Sasaki *et al.* 2004). Lines F, Hs and P accumulated more Al than CS (Fig. 4A, 4B), but they exhibited similar *ALMT1* expression (Fig. 4D) and comparable Al tolerance to that found in CS (Fig. 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, 1997b, 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.

We conclude that 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. 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.

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