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The impact of elevated CO_2 on acid-soil tolerance of hexaploid wheat (*Triticum aestivum* L.) genotypes varying in organic anion efflux

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Abstract:

Background & aim It is unclear how elevated CO_2 (eCO_2) affects the response of crops to soil acidity. This study examined the effect of eCO_2 on acid-soil tolerance of hexaploid wheat genotypes that vary in Al^{3+} resistance due to differences in root efflux of citrate and malate.

Methods Three pairs of near-isogenic lines were grown for 24-26 days under ambient CO_2 (400 ppm) and eCO_2 (800 ppm) in acid soils and hydroponics with various Al^{3+} concentrations. The lines consisted of pairs that differed in alleles of the *TaALMT1* and *TaMATE1B* genes. Plant growth parameters and rhizosphere soil properties were measured.

Results Elevated CO_2 increased the slope of negative correlations between root and shoot biomass and Al^{3+} concentration in the rhizosphere of a line that has an Al^{3+}-sensitive allele of the *TaALMT1* gene conditioning malate efflux (ES8), but did not change that of a near-isogenic sister line with an Al^{3+}-resistant *TaALMT1* allele (ET8). Elevated CO_2 decreased the relative root length and biomass (% of limed soil) of a line that lacked both malate and citrate efflux (Egret), but did not affect lines that possessed either malate or citrate efflux. Elevated CO_2 had no effect on either malate or citrate efflux from root tips of Al^{3+}-resistant lines. Elevated CO_2 also increased Al^{3+} concentration and decreased NH_4^+ concentration in rhizosphere soil, but decreased concentrations of Al and Zn in shoots.

Conclusions Elevated CO_2 decreased the acid-soil tolerance of Al^{3+}sensitive genotypes but not of Al^{3+} resistant genotypes. Malate efflux played a dominant role in conferring acid-soil tolerance to hexaploid wheat.

Key words: aluminium resistance, carbon allocation, genotypic variation, near-isogenic lines, nutrient mobilisation, root exudates

Introduction

Atmospheric CO_2 concentration has increased from 280 µmol mol^{-1} prior to the industrial revolution to a current level of 408 µmol mol^{-1} (https://www.co2.earth, March, 2018), and is predicted to be around 720 µmol mol^{-1} by the end of this century (IPCC 2014). Elevated CO_2 (eCO_2) generally enhances photosynthesis and plant growth, and hence plant productivity (Rogers et al. 1995). Elevated CO_2 can also increase the efflux of root exudates due to increased synthesis of carbon by shoots resulting in increased mobilisation of nutrients (Jin et al. 2014). The increased synthesis of carbon is also thought to alleviate heavy-metal toxicity (Jia et al. 2016) and to enhance plant tolerance to environmental stresses (Huang and Xu 2015).

Acid soils account for 40-50% of world arable land area, and are a serious constraint to root growth and crop production (Kochian et al. 2015). Furthermore, soil acidity increases as a consequence of agricultural production, during which cations are taken up by plants and exported as produce leaving behind acidity. In addition, soil also acidifies when nitrate (NO_3^-) derived from legume residues and ammonium-based fertilisers leaches beyond the rooting zone of crops and pastures (Guo et al. 2010; Hinsinger et al. 2003). When soil pH(H_2O) is less than 5.0, aluminium (Al^{3+}) becomes more soluble, and Al^{3+} toxicity becomes the primary limit to root elongation and thus plant growth (Kochian et al. 2015; Wright 1989). Many plant species have evolved mechanisms to detoxify Al^{3+} by either excluding Al^{3+} ion from roots (resistance) or by accumulating Al^{3+} safely in plant cells (tolerance), thus enhancing their ability to grow in acid soils (Ryan et al. 2011).

Currently characterized Al^{3+} detoxification genes in hexaploid wheat (*Triticum aestivum* L.) detoxify Al^{3+} through the resistance mechanism of organic anion (malate and citrate) efflux (Delhaize et al. 2012). Malate and citrate form complexes with Al^{3+}, detoxifying Al^{3+} in the apoplast or rhizosphere and increasing root and thus shoot growth in acid soils (Kopittke et al. 2017). There is a considerable genotypic variation in levels of Al^{3+} resistance within hexaploid wheat germplasm (Ryan et al. 1995a; Ryan et al. 2009) and genetic resistance to Al^{3+} has been conferred to sensitive genotypes by introgression of the *TaALMT1* (encoding for a transporter conferring malate efflux) and *TaMATE1B* genes (encoding for a transporter conferring citrate efflux) (Delhaize et al. 2012). Enhanced resistance has been observed in hydroponics and soil cultures (Delhaize et al. 1993b; Ryan et al. 2009; Sasaki et al. 2004; Tang et al. 2001) as well as in field trials (Pereira et al. 2015; Tang et al. 2002). Ryan et al. (2009) found that effluxes of malate and citrate from intact roots were comparable, whereas citrate efflux from excised apices was about a tenth of that found for malate. Malate efflux is activated by Al^{3+} while citrate efflux is constitutive, with both organic anions secreted primarily from the root apex. *TaALMT1* is more effective than *TaMATE1B* in conferring Al^{3+} resistance to hexaploid wheat, whereas the reverse was found in durum wheat (*Triticum durum*) grown in soil (Han et al. 2016). The effectiveness of the two mechanisms in conferring acid-soil tolerance to hexaploid wheat under different CO_2 regimes has not been directly compared.

There is a little existing information on the effect of eCO_2 on plants grown in acid soils with toxic concentrations of Al^{3+}. The only published study focusing on Al^{3+} resistance had demonstrated that eCO_2 did not affect the rates of malate efflux in excised root tips of either sensitive or resistant genotypes, indicating that Al^{3+} resistance did not change under eCO_2 (Tian et al. 2013). It is possible that eCO_2 decreases rhizosphere pH in Al^{3+}-toxic soils via the selective uptake of NH_4^+ over NO_3^- (Bloom et al. 2010; Carlisle et al. 2012), as has been demonstrated in the soils polluted by heavy metals (Li et al. 2013; Li et al. 2010; Wu et al.

2009), which could result in greater dissolution of Al^{3+}. If this were the case, the greater carbon fixed by shoots under eCO_2 might not be translated into increased root growth due to growth inhibition caused by the increased Al^{3+} toxicity. Elevated CO_2 enhanced plant tolerance to cadmium (Cd) and this was attributed to increased carbon fixation resulting in increased production of antioxidants (Guo et al. 2015; Jia et al. 2010). It is possible that eCO_2 might enhance the Al^{3+} tolerance of wheat via a similar mechanism. Elevated CO_2 also promotes the efflux of root exudates, such as soluble sugars, organic acids, and amino acids (Johansson et al. 2009; Phillips et al. 2011). Al^{3+}-resistant wheat genotypes exude more organic anions than sensitive genotypes and eCO_2 might further increase organic anion efflux to increase their tolerance of acid soils.

This study extended the experiments described by Tian et al. (2013) by using three pairs of near-isogenic lines (NILs) of wheat differing in Al^{3+} resistance. In addition, we report the effect of eCO_2 on the same pair of lines used by Tian et al. (2013) when grown in a series of soils with graduated levels of Al^{3+}-toxicity. In another experiment, the germplasm included lines that secreted citrate constitutively either in the presence or absence of the *TaALMT1* gene responsible for malate efflux (Han et al. 2016). The aim was to test the hypothesis that eCO_2 would increase the acid-soil tolerance of resistant genotypes relative to sensitive genotypes when grown in acid soils. According to this hypothesis, the relative growth of resistant genotypes in response to eCO_2 would be greater than that of sensitive genotypes. This study also compared the relative performance of genotypes exuding either malate or citrate or both organic anions in an acid soil.

Materials and methods

Germplasm

The lines used in the experiments are summarised in Table 1. ES8 and ET8 are NILs that differ at the major locus for Al^{3+} resistance in hexaploid wheat (Delhaize et al. 1993a). *TaALMT1* underlies the Al^{3+} resistance locus and confers the Al^{3+}-activated malate efflux from root apices that is responsible for resistance (Delhaize et al. 2012). The other NILs differ for the *TaMATE1B* gene and were developed by backcrossing cv. Carazinho (donor of the Al^{3+}-resistant *TaMATE1B* allele) to cultivars Egret and EGA-Burke (Han et al. 2016). After either six (cv. Egret) or nine (cv. EGA-Burke) backcrosses, single plants in the F_2 generation that differed for the *TaMATE1B* allele were selected and used to develop sister lines. The cv. Egret possesses the Al^{3+}-sensitive allele of *TaALMT1* (low malate efflux) whereas cv. EGA-Burke has the Al^{3+}-resistant allele of *TaALMT1* (high malate efflux).

Experimental design and plant culture

Experiment 1

Experiment 1 consisted of two CO_2 levels, two wheat genotypes and five composite soils varying in Al^{3+} toxicity in a blocked split-plot design with CO_2 as the main plot, and genotypes by soils as the subplot. The CO_2 treatments were maintained at 400 ± 15 (aCO_2) and 800 ± 30 (eCO_2) µmol mol^{-1} in four growth chambers (Fitotron® SGC120, Weiss Technik, UK) and each CO_2 level had two replicated chambers, with one replication as a block to minimise the variation due to potential differences between chambers. Within each chamber, each subplot had two replicates. The two wheat genotypes were Al^{3+}-sensitive ES8 and its near isogenic pair Al^{3+}-resistant ET8. Five experimental soils were made by mixing different ratios of a Dermosol and a Ferrosol (Isbell 1996), which both consisted of 0-0.2 m topsoil collected from two sites in Kinglake National Park, VIC, Australia (Table 2). A preliminary experiment showed that Al concentrations in the Dermosol were too toxic to allow sufficient growth of ES8, therefore the five soil treatments consisted of an 80:20

(Dermosol: Ferrosol) ratio mix, a 60:40 ratio mix, a 40:60 ratio mix, a 20:80 ratio mix and a Ferrosol (0:100), which generated composite soils that varied in Al^{3+} concentration. One kilogram of experimental soil (passed through a 2-mm sieve) was mixed with the following basal nutrients (mg kg^{-1} soil): K_2SO_4, 147; $MgSO_4.7H_2O$, 122; $CaCl_2.2H_2O$, 186; KH_2PO_4, 112.5; $CO(NH_2)_2$, 400; $CuSO_4.5H_2O$, 6; $ZnSO_4.7H_2O$, 8; $NaMoO_4.2H_2O$, 0.4; and $NaB_4O_7.10H_2O$, 1.6. The soils were then placed in PVC pots 250 mm in height and 75 mm in diameter. Twelve pre-germinated (2 d at 25 ˚C) and uniform seeds were planted in each pot. The plants were placed in growth chambers and the ambient temperature was set at 22˚C for the 14-h light/day period and 18˚C for the 10-h dark/night period. The humidity was set at 70%. Light intensity measured at the surface of the pots during the day period was 300 µmol m^{-2} s^{-1} photon irradiance. Seedlings were thinned to six plants per pot 6 d after sowing. The pots were watered to 80% of field capacity by weight every 2 d. Plants were grown for 25 d from sowing until harvest.

Experiment 2

Experiment 2 was also a blocked split-plot design and consisted of two CO_2 levels as the main plot, and six wheat genotypes by two soils as the sub-plot. The six genotypes are listed in Table 1. The untreated Dermosol was used to impose severe Al toxicity to the genotypes. The other soil was the same Dermosol with 4 g kg^{-1} soil of lime ($CaCO_3$) added, which increased the pH from 4.1 to 4.8 and $CaCl_2$-extractable Al from 53.8 to 1.0 mg kg^{-1} after three weeks' incubation. Nitrogen and K concentration in the basal nutrients were changed to 60 mg urea kg^{-1} soil and 441 mg K_2SO_4 kg^{-1} soil. Other experimental practices were the same as Experiment 1. Plants were grown for 24 d from sowing to harvest.

Experiment 3

Experiment 3 consisted of two CO_2 levels as the main plot as for Experiments 1 and 2, and one near-isogeneic pair (EGA-Burke and EGA-Burke *TaMATE1B*) as the sub-plot using the same growth chambers as before**.** The hydroponic study was conducted using 40-L nutrient solution in each rectangular container (650 mm × 390 mm × 240 mm), which contained the following nutrients in µM: KNO_3, 1000; NH_4Cl, 500; 10 or 100 KH_2PO_4, $CaCl_2$, 500; $MgSO_4$, 150; $FeSO_4$, 10; H_3BO_3, 11; $MnCl_2$, 2; $CuCl_2$, 0.2 and $ZnCl_2$, 0.35. Wheat seeds were surface-sterilized in 0.5% sodium hypochlorite (v/v) for 20 min, rinsed twice with reverse osmosis water and germinated at 25 ˚C in the dark for 2 d. After germination, seedlings were transferred to the nutrient solution containing 100 µM P and grown for 5 d, and then transplanted to the nutrient solution containing 10 µM P when the CO_2 treatment commenced. The solution was continuously aerated and its pH adjusted to 4.30 using 0.5 M HCl or 0.5 M NaOH at least twice a day. The solution was renewed weekly. The root tips were harvested for measurement of organic anions at 24 days after germination.

Plant harvest and measurements

For the pot experiments (Experiments 1 and 2) at harvest, shoots were cut at soil level, rinsed twice in 0.1 M HCl (Tang et al. 1990) and then rinsed twice in reverse osmosis (RO) water to remove dust and other particulates. One portion of the roots was carefully washed with RO water twice. All plant materials were dried at 70 ˚C for at least 72 h and then weighed. Shoot materials were digested in concentrated nitric acid (HNO_3) in 50-ml Eppendorf reaction vials using a microwave reactive system (Multiwave 3000, Anton Paar GmbH, Austria). All samples were diluted and analysed for Al, Mn, and Zn concentrations using inductively coupled plasma atomic emission spectrometry (ICP-AES, Optima 8000, PerkinElmer, US). Another portion of the fresh roots was floated in RO water in a clear perspex tray and

scanned using a flatbed scanner (EPSON EU-35, Seiko Epson Corp., Japan) at 600 dots per inch. Root length and diameter were determined using the WinRhizo Proversion Version 2003B software (Régent Instruments Inc., Canada) before being dried and weighed. Since the least Al^{3+}-toxic soil used in Experiment 1 (Ferrosol) still inhibited root growth of sensitive genotypes, acid-soil tolerance (relative growth, %) was not calculated. In Experiment 2, the acid-soil tolerance of genotypes was assessed using the ratio of plant growth (including root length, root and shoot biomass) in toxic soil to that in control limed soil to minimize the impacts of plant size on acid tolerance.

Organic anion measurements

The efflux of organic anions from root tips was measured for plants grown in nutrient solution (Experiment 3). The 0-5 mm tips of the primary roots (30 tips per replicate) were excised, placed in 5-ml glass vials. The root tips were immersed with 1.0 ml 0.2 mM $CaCl_2$ (0.22 µm-filter sterilised, pH 4.30) and shaken horizontally (70 rpm) for 1 h at 18 ˚C to remove any organic anions released from the excised surface. Root tips were rinsed again with 1.0 ml of 0.2 mM $CaCl_2$. The solutions were replaced by 1.0 ml of 0.2 mM $CaCl_2$ containing 200 µM Al^{3+} (0.22 µm-filter sterilised, pH 4.30) and returned to the shaker for a 1.5 h-incubation (Ryan et al., 1995b).

The concentrations of organic anions in the solution were determined using liquid chromatography mass spectrometry (LC-MS, HPLC, 1290 infinity, Agilent technologies, and MS, Orbitrap Elite, Thermo Scientific, US). HPLC separation was achieved using a Rezex ROA-organic anion column (150 × 4.6 mm, Phenomenex, US) on an Agilent 1290 infinity UPLC system, equipped with degasser, a binary pump, a temperature-controlled autosampler at 8 ˚C and a column compartment at 30 ˚C. The mobile phase for the separation was 0.5% formic acid. An isocratic elution at a flow rate of 0.3 ml min^{-1} was adopted (total run time 10 min) and the injection volume was 5 µl for all analyses.

Soil measurements

Pots from Experiments 1 and 2 were disassembled and roots were gently shaken, such that the bulk soil fell off and the soil left adhering to the root surface was collected as rhizosphere soil. Soil solution was extracted with 0.01 M $CaCl_2$ (soil: solution, 1:5) using 5 g fresh rhizosphere soil. After end-over-end mixing for 1 h, the suspension was centrifuged at 3000 rpm for 5 min then the soil extracts were filtered through a 0.22-µm membrane. The solution pH was measured and Al concentration of the filtered extracts was spectrophotometrically measured with the pyrocatechol violet method, referred to here as PCV-Al (Kerven et al. 1989). Ammonium (NH_4^+) concentrations in the extracts were determined using a flow injection analyser (QuickChem 8500, LACHAT, US). The microbial respiration rates of the rhizosphere soil were determined using an infrared gas analyser (Servomex 4210, Servomex, UK) after 24-h incubation at 25 ˚C in dark (Jin et al. 2014).

Statistical analysis

The data were statistically analysed using analysis of variance (ANOVA) assuming split-plot designs to determine the effects of CO_2, genotype and their interactions by using GenStat for Windows (Version 17.1, VSN International, UK). Least significant difference tests (LSD) were used to assess the differences between means ($p=0.05$). Linear regression analysis of the relationships between PCV-Al in rhizosphere soil and root length, root biomass or shoot biomass was used to test for differences in relationships between CO_2 treatments using GenStat. A two-tailed *t*-test was performed using Microsoft Excel 2016 to compare acid-soil tolerance between two CO_2 treatments.

Results

Plant growth

In Experiment 1 that compared growth of ES8 and ET8 under two CO_2 treatments, the response of plant height and water use to the CO_2 treatments had occurred around 2 weeks (Fig. S1). After 25 d growth, the linear regressions of PCV-Al concentration with root length, root biomass and shoot biomass of ES8 and ET8 were all significant (p<0.001, Fig. 1). There were no significant differences of gradients (p=0.268) and intercepts (p=0.291) between eCO_2 and aCO_2 in linear functions fitted to root length of ES8. However, eCO_2 decreased the gradients of functions fitted to shoot biomass (p=0.041) and root biomass (nearly significant, p=0.062) of ES8 (steeper gradients), whilst it increased the intercepts of functions fitted to root biomass (p=0.035) and shoot biomass (p=0.038). By contrast, eCO_2 had no significant effects on the gradients of functions fitted to root length, root biomass and shoot biomass of ET8, but it increased the intercepts of functions fitted to root length (p=0.022) and root biomass (p<0.001) and decreased that of shoot biomass (p=0.018).

In Experiment 2, all six genotypes were grown and eCO_2 had no main effect on root length, biomass and diameter, or root-to-shoot ratio in the acid soil, but it increased shoot biomass by 16% on average (p<0.001) and there were significant interactions with genotype (Table 3). Specifically, eCO_2 increased root biomass of ET8, EGA-Burke and EGA-Burke *TaMATE1B* but did not affect that of ES8, Egret and Egret *TaMATE1B*. There was a similar pattern of response in root length, but the effect was only significant in ET8. Elevated CO_2 increased shoot biomass of EGA-Burke and EGA-Burke *TaMATE1B* more than that of other genotypes. It decreased root-to-shoot ratio and increased root diameter of Egret, with no impact on root-to-shoot ratio and root diameter of other genotypes. The six genotypes differed substantially in their root length, root biomass and shoot biomass responses to soil acidity (p<0.001, Table 3). On average, in this acid soil, EGA-Burke *TaMATE1B* had the greatest root length, followed by EGA-Burke, ET8, Egret *TaMATE1B*, ES8, and Egret, with the difference between the greatest and smallest being 8-fold. Compared with their NILs, ET8, Egret *TaMATE1B* and EGA-Burke *TaMATE1B* increased root length by 236%, 85% and 11%, and root biomass by 46%, 32% and 11%, respectively.

Relative growth

In Experiment 2, although the main effect of CO_2 and CO_2 × genotype interactions were not significant for relative root length, root biomass and shoot biomass, eCO_2 decreased the relative root length of Egret from 15% to 11% (p=0.030) (Table 4). There was a significant genotypic variation in response to the acid soil (p<0.001), with EGA-Burke and EGA-Burke *TaMATE1B* having the greatest relative root length, root biomass and shoot biomass, followed by Egret *TaMATE1B*, ET8, ES8 and Egret the lowest (Table 4).

Concentrations of Al, Mn, and Zn in shoots

In Experiment 2, eCO_2 decreased Al concentration in shoots by an average of 24% in the acid soil (p<0.001) (Table 5). ES8 had the highest Al concentration, followed by ET8, Egret, Egret *TaMATE1B* and EGA-Burke while EGA-Burke *TaMATE1B* the lowest. The decrease in Al concentration was less in EGA-Burke and EGA-Burke *TaMATE1B* than in other genotypes, resulting in significant CO_2 × genotype interaction. The exponential least squares regression showed that the Al concentration in shoots was closely related to shoot biomass (r=-0.82, p<0.001).

Elevated CO_2 did not affect Mn concentration but decreased Zn concentration in shoots by 7% (p=0.009) whilst there were no significant CO_2 × genotype interactions on Mn and Zn concentration. There were main effects of genotype on shoot Mn, and Zn concentration when grown in acid soil (p<0.001). In general, EGA-Burke *TaMATE1B* had the highest Mn and Zn concentrations, followed by EGA-Burke, ET8, Egret *TaMATE1B*, ES8, and Egret the lowest. Specifically, compared with their NILs, ET8, Egret *TaMATE1B* and EGA-Burke *TaMATE1B* had 40%, 27% and 7% higher Mn concentration in shoots, and ET8 and Egret *TaMATE1B* had 36% and 16% higher Zn concentration, respectively.

Soil properties

In Experiment 1, there were no interactions between CO_2 and soil on rhizosphere pH, PCV-Al concentration, NH_4^+ concentration, and microbial respiration (Table S1). Logarithmic least squares regression showed a close correlation between the rhizosphere Al concentration and the rhizosphere pH (r= -0.97, p<0.001). Although eCO_2 had no main effect on rhizosphere pH and microbial respiration (Table 6), it tended to increase rhizosphere PCV-Al concentration (p=0.070), and decreased NH_4^+ concentration by an average of 15% (p<0.001). There were no significant CO_2 × genotype interactions on rhizosphere pH, PCV-Al and microbial respiration. However, eCO_2 decreased NH_4^+ concentration more in ET8 than ES8 rhizosphere. As the main effect of genotype, ET8 (relative to ES8) increased rhizosphere soil respiration, and lowered the NH_4^+ concentration and rhizosphere pH which corresponded with increased PCV-Al concentrations.

In Experiment 2, there was no main effect of CO_2 nor CO_2 × genotype interactions on rhizosphere pH and PCV-Al concentration in the rhizosphere (data not shown).

Organic anions

Experiment 3 examined the effect of eCO_2 on the efflux of organic anions for EGA-Burke and EGA-Burke TaMATE1B. By using the EGA-Burke isogenic lines, we were able to assess the effect of eCO_2 on both malate and citrate efflux. When averaging all data, eCO_2 did not affect the efflux rates of either malate or citrate of both genotypes with EGA-Burke *TaMATE1B* releasing more citrate (Figure 2).

Discussion

The effects of eCO_2 on acid-soil tolerance

Elevated CO_2 decreased the acid-soil tolerance of sensitive genotypes ES8 and Egret. This was demonstrated by the observed eCO_2-stimulated steeper gradients of linear functions fitted to root and shoot biomass of ES8 and PCV-Al in rhizosphere (Fig. 1b and c) and the decrease of relative root length of Egret in Experiment 2 (Table 4). The decrease in root length of ES8 in Experiment 1, particularly in highly toxic soils, and the tendency for decreases in the root-to-shoot ratio and increases in root diameter of ES8 and Egret in both experiments under eCO_2 (Tables 3 and S2) indicate that eCO_2 limited root growth by exacerbating root Al^{3+} toxicity. The decrease in the root-to-shoot ratio also indicates that eCO_2 promoted biomass allocation to shoots rather than roots, which may explain why eCO_2 had no effect on relative shoot biomass of ES8 and Egret in Experiment 2. Our study is inconsistent with several reviews showing eCO_2 increases root growth and biomass as a general phenomenon (Madhu and Hatfield 2013; Wang et al. 2013). The discrepancy between our study and previous studies for the sensitive lines can be attributed to the use of acid soils in our study where Al^{3+} toxicity counteracted the positive effect of eCO_2 on plant growth (Table S3, Rogers et al. 1995; Wang et al. 2013).

By contrast, resistant genotypes maintained their tolerance in the acid soils under eCO_2. The relative root length, relative root biomass and relative shoot biomass of ET8, Egret *TaMATE1B*, EGA-Burke and EGA-Burke *TaMATE1B* were not affected by eCO_2 (Table 4). The maintenance of acid-soil tolerance of resistant genotypes therefore can benefit their root elongation (Table 3). Tian et al (2013) showed eCO_2 increased the root elongation and biomass of ET8 more than that of ES8 in an acid soil in open top chambers, which is consistent with our results.

Several studies have demonstrated that eCO_2 significantly increased toxic metal availability in metal-polluted soils (Guo et al. 2011; Jia et al. 2014; Li et al. 2013), which is also consistent with the data for extractable soil Al in our study. Inhibition of root elongation and reduced root-to-shoot ratio of sensitive genotypes, particularly in highly toxic soil (Fig. 1, Tables 3 and S2) was consistent with our observations of eCO_2 increasing soil Al^{3+} concentration (Table 6). The likely mechanism for this was the enhancement of NH_4^+ uptake (Table 6), which likely acidified the rhizosphere soil to dissolve additional Al^{3+}. This theory is further supported by observed differences in behaviour of ES8 and ET8 between Experiments 1 and 2. In Experiment 1, a higher concentration of urea was applied, which would have made more NH_4^+ available to plants, and eCO_2 increased root elongation of ET8 to a lesser extent and inhibited root elongation of ES8 more in comparison to Experiment 2. A greater number of yellow leaf tips (likely Al toxicity symptoms in shoots) was also observed in ES8 in the most Al^{3+}-toxic soil in Experiment 1 than in Experiment 2 (data not shown). Furthermore, eCO_2 increased rhizosphere Al concentration in Experiment 1 but not in Experiment 2. Therefore, we propose that eCO_2 promotes shoot growth, which in turn increases NH_4^+ uptake and hence rhizosphere acidification, leading to mobilisation of Al^{3+} into the soil solution.

Whilst higher microbial respiration rates of the rhizosphere soil (Table 6) are consistent with plants grown under eCO_2 having more root exudates as has been demonstrated in other studies (Jia et al. 2014; Johansson et al. 2009), a greater efflux of root exudates under eCO_2 in our experiments, if there was any, did not help enhance the acid-soil tolerance of resistant genotypes. Despite a possible increase in root exudates by eCO_2, there was no matching decrease in rhizosphere PCV-Al concentration by formation of exudate-Al^{3+} complexes or the effects of root exudates were eliminated by rhizosphere acidification. Also, organic anion efflux can promote shoot uptake of divalent ions such as Mn and Zn by protecting root growth or stimulating ion mobilization in soils (Khabaz-Saberi et al. 2010; Scott et al. 1998; Widodo et al. 2010). When compared with corresponding NILs, the greater concentrations of Mn and Zn in shoots were closely related to the lines that possessed malate or citrate efflux (Table 5). The decreases in shoot Mn and Zn concentrations in EGA-Burke and EGA-Burke *TaMATE1B* by lime addition further highlighted the possibility of Mn and Zn mobilization in soils through malate efflux (Tables 5 and S4) since lime would have decreased the Al^{3+} bioavailability and eliminated malate efflux.

Elevated CO_2 had no main effect nor interactions with genotype on Mn accumulation in shoots, but decreased Zn accumulation in shoots, indicating that eCO_2 did not affect or might have even reduced malate or citrate efflux. This conclusion is further supported by the general maintenance of the efflux of both malate and citrate in EGA-Burke and EGA-Burke *TaMATE1B* in our hydroponic experiment (Figure 2). Direct measurement using excised root tips of ET8 and ES8 (Tian et al. 2013) found that eCO_2 did not affect malate efflux, which is consistent with our study. Since organic anion efflux depends on transport across plasma membranes rather than synthesis of the compounds (Delhaize et al. 2012), it is less likely that

the increased carbon fixation in leaves under eCO_2 can affect the expression or activity of these transporters in root tips. Although other researchers have speculated that increased root exudates under eCO_2 could protect root growth from metal toxicity (Jia et al. 2016; Li et al. 2014), our results showed that eCO_2 was unlikely to have promoted the efflux of specific root exudates to overcome rhizosphere acidification in hexaploid wheat. We propose that the maintenance of acid-soil tolerance in resistant genotypes can be attributed to greater carbon fixation, thus greater antioxidant capacity in shoots under eCO_2 compared with sensitive genotypes (Guo et al. 2015; Jia et al. 2010).

Genotypic variation in growth responses to acid soils

This study confirmed that the introgression of the *TaALMT1* gene into sensitive genotypes of hexaploid wheat enhanced the acid-soil tolerance more than *TaMATE1B* gene in highly Al^{3+}-toxic soils as demonstrated by Han et al. (2016). Introgression of the resistant allele of the *TaALMT1* gene (ET8 compared to ES8) conferred a greater enhancement of its relative root length, root length, and root biomass than introgression of a resistant allele of the *TaMATE1B* gene (Egret *TaMATE1B* vs. Egret) (Tables 3 and 4). The other piece of evidence was a similar pattern of shoot Mn and Zn accumulation in shoots and root length among the genotypes (Table 5). The greater shoot Mn and Zn accumulation by the lines that possess malate efflux compared to citrate efflux indicates that the direct malate effect of binding and detoxifying Al^{3+} might be greater than citrate in hexaploid wheat due to its higher concentration in root apices (Ryan et al. 2009), but we cannot rule out the possibility that it is the secondary effect of better root growth. The additive effect of the *TaMATE1B* gene with *TaALMT1* in EGA-Burke on root growth in acid soils in our study confirmed the observation by Han et al. (2016) in nutrient solution. Compared with EGA-Burke, the increase of shoot Mn concentration of EGA-Burke *TaMATE1B* in either limed or unlimed soil under eCO_2 conditions is likely to be due to the efflux of citrate (Tables 5 and S4).

Conclusion

This study demonstrated that eCO_2 decreased acid-soil tolerance of sensitive genotypes but did not affect the tolerance of resistant genotypes. Elevated CO_2 increased rhizosphere acidification and hence Al^{3+} bioavailability probably through increased uptake of NH_4^+. It appears that rhizosphere acidification under eCO_2 did not reduce root elongation of resistant genotypes due to unknown mechanisms. Elevated CO_2 did not affect malate and citrate efflux in EGA-Burke and EGA-Burke *TaMATE1B*. By using NILs of wheat that vary in organic anion efflux, the study also confirmed that malate plays the dominant role in enhancing acid-soil tolerance in hexaploid wheat. Given that both atmospheric CO_2 concentration and soil acidity will continue to increase, it is prudent for wheat breeders to maintain Al^{3+} resistance genes in their germplasm, as these will become increasingly important for maintaining future crop production on acid soils.

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There are four supplementary tables and one figure associated.

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560

561 **Table 1**. The three pairs of wheat genotypes used in the study, their specific root exudation of
562 malate and citrate (-, low exudation; +, high exudation) and classification of their Al^{3+}
563 resistance.
564

Genotypes	Pairing	Root exudations		Al^{3+} resistance	References
		Malate	Citrate		
ES8	Isogenic pairs	-	-	Low	(Delhaize et al. 1993a)
ET8		+	-	High	
Egret	Isogenic pairs	-	-	Low	(Han et al. 2016)
Egret *TaMATE1B*		-	+	Moderate	
EGA-Burke	Isogenic pairs	+	-	High	(Han et al. 2016)
EGA-Burke *TaMATE1B*		+	+	High	

565 **Table 2.** Sampling location and basic chemical properties of experimental soils used in this
566 study.

Soil	Collection sites	pH ($CaCl_2$)	Total C (g kg^{-1})	Total N (g kg^{-1})	PCV-Al [a] (mg kg^{-1})	ICP-Al [b] (mg kg^{-1})	Olsen-P [c] (mg kg^{-1})	Colwell-P [c] (mg kg^{-1})	PBI [c] (mg P kg^{-1})
Dermosol	37.462S, 145.263E	4.12	44.2	2.32	42.6	53.8	7.3	9.5	659
Ferrosol	37.474S, 145.257E	4.55	58.8	3.13	5.1	14.8	5.7	8.6	671

567 a. Concentrations of extractable Al (0.01 M $CaCl_2$) in the soils were measured with the
568 pyrocatechol violet (PCV) method (Kerven et al. 1989).
569 b. Concentrations of extractable Al (0.01 M $CaCl_2$) in the soils were measured by ICP-AES.
570 c. Measurements of Olsen-P, Colwell-P and PBI (phosphorus buffer index) were referred to
571 Rayment and Lyons (2011).

572 **Table 3**. Root length, root and shoot biomass, ratio of root to shoot biomass (root/shoot) and root diameter of six wheat genotypes grown for 24
573 d in the Dermosol (pH = 4.12) under two CO_2 concentrations (aCO_2, 400 µmol mol^{-1} and eCO_2, 800 µmol mol^{-1}) (Experiment 2)
574

Genotypes	Root length (m $plant^{-1}$)			Root biomass (mg $plant^{-1}$)			Shoot biomass (mg $plant^{-1}$)			Root/shoot			Root diameter (µm)		
	aCO_2	eCO_2	Means	aCO_2	eCO_2	Means	aCO_2	eCO_2	Means	aCO_2	eCO_2	Means	aCO_2	eCO_2	Means
ES8	1.9	1.9	1.9	40	44	42	35	38	37	1.13	1.09	1.11	488	507	498
ET8	5.7	7.2	6.5	56	66	61	43	47	45	1.31	1.40	1.36	325	326	326
Egret	1.6	1.3	1.5	42	42	42	35	43	39	1.20	0.98	1.09	561	598	580
Egret *TaMATE1B*	2.7	2.6	2.7	55	55	55	41	45	43	1.36	1.24	1.30	498	517	508
EGA-Burke	9.6	10.3	10.0	80	93	87	63	75	69	1.26	1.23	1.25	317	330	324
EGA-Burke *TaMATE1B*	10.8	11.3	11.1	89	103	96	70	84	77	1.26	1.24	1.25	309	315	312
Means	*5.4*	*5.8*		*60*	*67*		*48*	*55*		*1.25*	*1.20*		*416*	*432*	
p-value (LSD, *p=0.05*)															
CO_2	0.254			0.202			<0.001 (1)			0.327			0.148		
Genotype	<0.001 (0.6)			<0.001 (4)			<0.001 (4)			<0.001 (0.08)			<0.001 (18)		
CO_2 × Genotype	0.037 (0.8)			0.003 (9)			0.046 (5)			0.017 (0.14)			0.408		

Table 4. Relative root length (a), relative root biomass (b), and relative shoot biomass (c) (as % of the limed at 4 g $CaCO_3$ kg^{-1}) of six wheat genotypes grown for 24 d in the Dermosol (pH = 4.12) under two CO_2 concentrations (400 µmol mol^{-1} and 800 µmol mol^{-1}) (Experiment 2)

Genotypes	Relative root length (%)			Relative root biomass (%)			Relative shoot biomass (%)		
	aCO_2	eCO_2	Means	aCO_2	eCO_2	Means	aCO_2	eCO_2	Means
ES8	23	18	20	64	58	61	67	64	65
ET8	62	67	65	78	81	79	71	74	72
Egret	15	11*	13	59	51	55	61	64	63
Egret *TaMATE1B*	26	22	24	78	68	73	68	70	69
EGA-Burke	82	77	80	92	93	93	76	82	79
EGA-Burke *TaMATE1B*	87	81	84	91	96	94	78	82	80
p-value (LSD, *p=0.05*)									
CO_2	0.466			0.439			0.332		
Genotype	<0.001 (6)			<0.001 (7)			<0.001 (7)		
CO_2×Genotype	0.121			0.247			0.915		

* indicates the significant difference between two CO_2 treatments at $p<0.05$ by using a two-tailed *t*-test.

578 **Table 5.** The concentrations of Al, Mn and Zn in shoots of six wheat genotypes grown for 24
579 d in the Dermosol (pH = 4.12) under two CO_2 concentrations (aCO_2, 400 µmol mol^{-1} and
580 eCO_2, 800 µmol mol^{-1}) (Experiment 2)

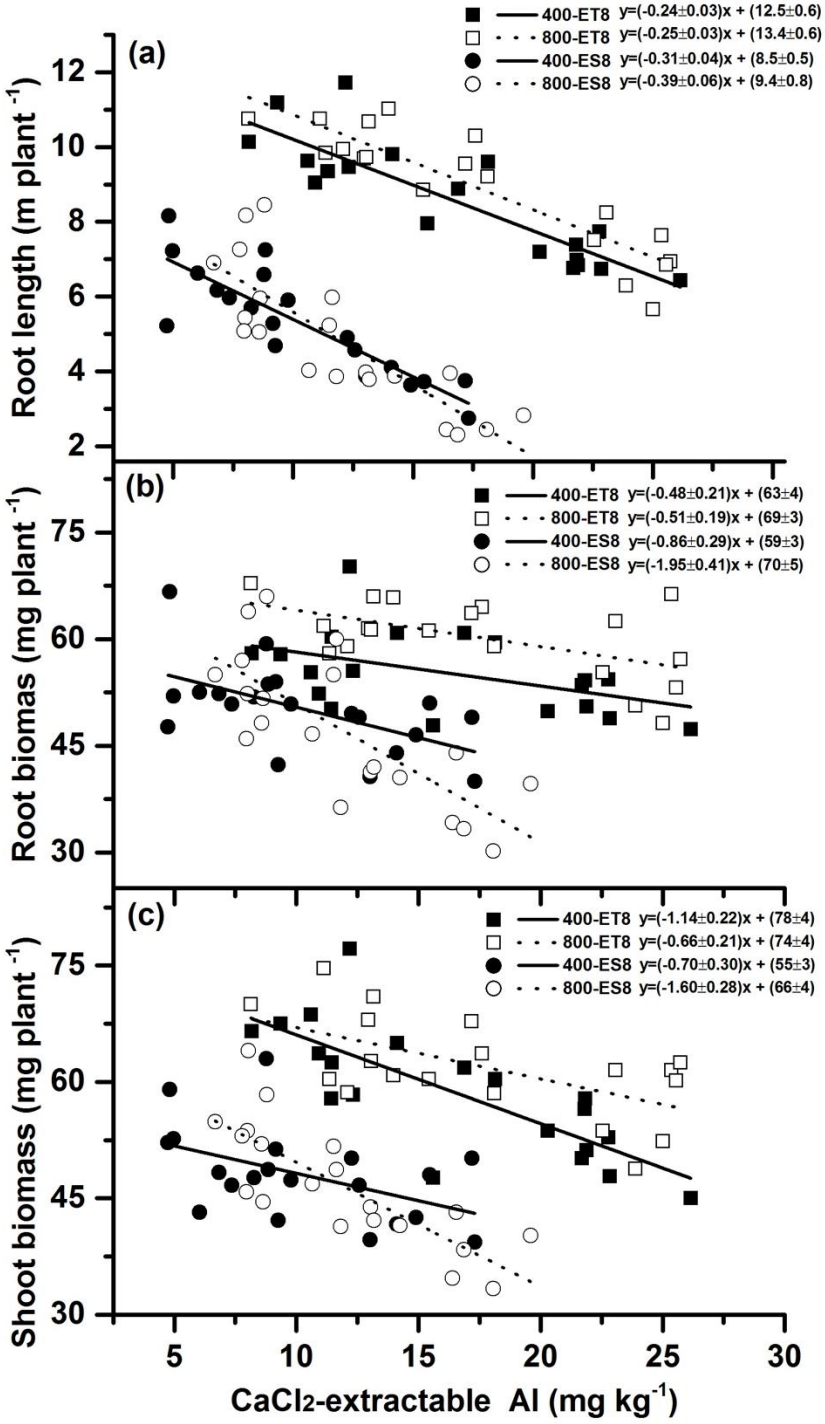
Genotypes	Al (µg g^{-1})			Mn (µg g^{-1})			Zn (µg g^{-1})		
	aCO_2	eCO_2	Means	aCO_2	eCO_2	Means	aCO_2	eCO_2	Means
ES8	166	134	150	90	96	93	25.6	22.9	24.3
ET8	165	102	134	129	131	130	34.9	30.9	32.9
Egret	151	108	130	93	82	88	18.3	14.9	16.6
Egret *TaMATE1B*	131	100	116	111	111	111	19.5	19.2	19.4
EGA-Burke	95	86	91	169	162	166	45.7	45.4	45.6
EGA-Burke *TaMATE1B*	88	72	80	173	180	177	46.8	44.6	45.7
Means	*132*	*102*		*127*	*127*		*31.8*	*29.6*	
p-value (LSD, *p=0.05*)									
CO_2	<0.001 (8)			0.820			0.009 (1.5)		
Genotype	<0.001 (14)			<0.001 (10)			<0.001 (2.6)		
CO_2 × Genotype	0.010 (20)			0.368			0.536		

581

582 **Table 6.** The pH, concentrations of $CaCl_2$-extractable Al (PCV-Al) and ammonium (NH_4^+),
583 and microbial respiration of rhizosphere soil with ES8 and ET8 grown for 25 d under two
584 CO_2 concentrations (aCO_2, 400 µmol mol^{-1} and eCO_2, 800 µmol mol^{-1}) (Experiment 1)

Genotypes	pH		PCV-Al (mg kg^{-1})		NH_4^+ (mg kg^{-1})		Respiration (ng C g^{-1} soil h^{-1})	
	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
ES8	4.380	4.364	10.3	11.9	114	105	5.47	5.98
ET8	4.293	4.284	16.5	18.4	76	52	6.18	7.28
p-value (LSD, *p*=0.05)								
CO_2	0.252		0.070		<0.001 (3)		0.182	
Genotype	<0.001 (0.007)		<0.001 (0.4)		<0.001 (3)		<0.001 (0.3)	
CO_2×Genotype	0.323		0.195		<0.001 (4)		0.079	

585 Values are means (n= 20) and least significant difference values (LSD) are given only when the *p*-
586 value is <0.05. The data from various soil treatments were combined as there was no interactions
587 between CO_2 and soil nor three-way interaction on these parameters (Table S1).



588

589 **Figure 1.** The relationships between $CaCl_2$-extractable Al (PCV-Al) in rhizosphere and root
590 length (a), root biomass (b) and shoot biomass (c) of ES8 and ET8 grown for 25 d in the soils
591 with various levels of Al toxicity (ratios of a Dermosol and a Ferrosol mixture: 0:100, 20:80,
592 40:60, 60:40 and 80:20) under two CO_2 concentrations (aCO_2, 400 µmol mol^{-1} and eCO_2, 800
593 µmol mol^{-1}). The values of the equation given were gradients/intercepts ± s.e. (Experiment
594 1).

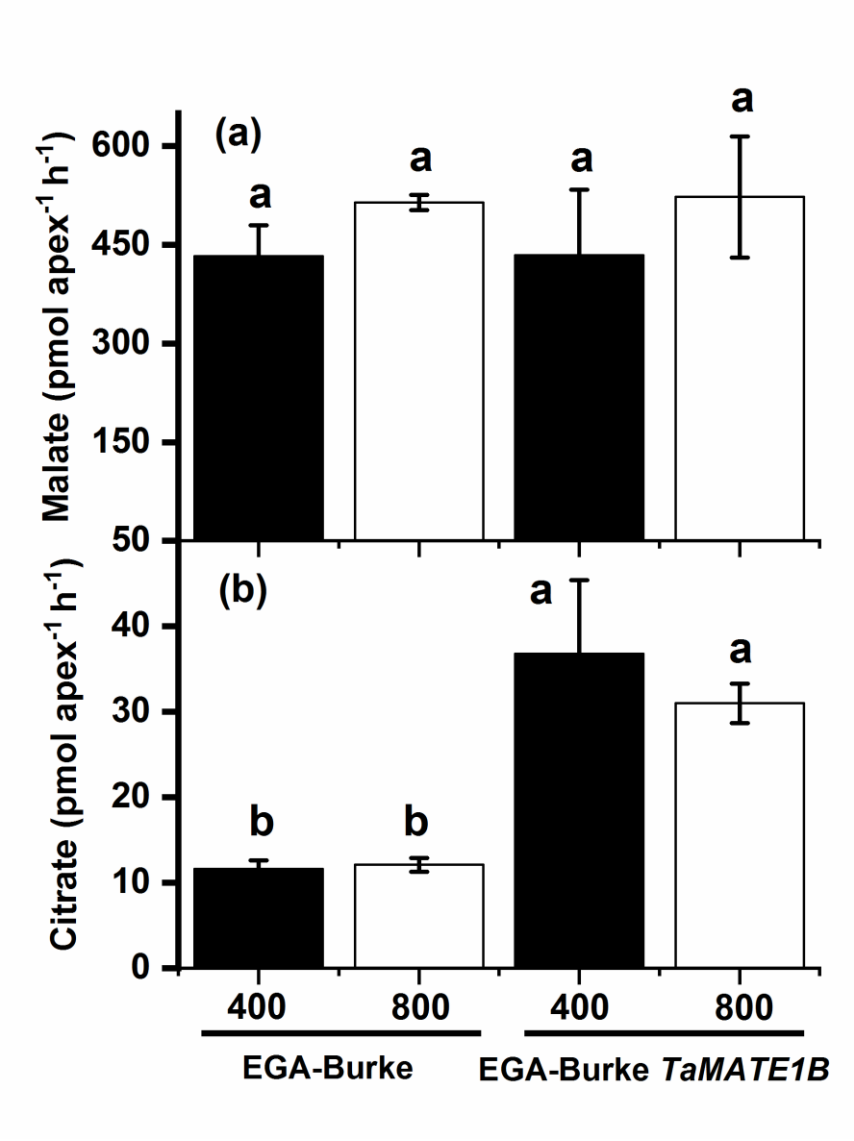


Figure 2. Malate (a) and citrate (b) efflux in 0-5 mm root tips of EGA-Burke and EGA-Burke *TaMATE1B* grown for 24 d under two CO_2 concentrations (400 and 800 µmol mol^{-1}). The same letter indicates the differences between two means are not significant (*p=0.05*) using least significant difference tests. Data are means ± se (n=4) (Experiment 3).

Supplementary information

The impact of elevated CO_2 on acid-soil tolerance of hexaploid wheat (*Triticum aestivum* L.) genotypes varying in organic anion efflux

By Dong et al.

Table S1. Soil pH, $CaCl_2$-extractable Al concentration (PCV-Al), ammonium (NH_4^+) and microbial respiration of rhizosphere soil with ES8 and ET8 grown for 25 d in the soils with various levels of Al toxicity under two CO_2 concentrations (aCO_2, 400 µmol mol^{-1} and eCO_2, 800 µmol mol^{-1}). Various levels of Al toxicity ($CaCl_2$-extractable Al concentrations of 4.4, 5.8, 7.8, 11.8, 16.5 mg kg^{-1}, respectively) were created by mixing a Dermosol and a Ferrosol at ratios of 0:100, 20:80, 40:60, 60:40 and 80:20 (Experiment 1).

Genotypes	Soils	pH		Al (mg kg^{-1})		NH_4^+ (mg kg^{-1})		Respiration (ng C g^{-1} soil h^{-1})	
		aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
ES8	0:100	4.481	4.438	5.1	7.8	87	81	3.25	2.55
	20:80	4.428	4.416	8.0	8.3	93	102	1.83	1.65
	40:60	4.402	4.368	9.4	11.4	105	109	0.68	0.92
	60:40	4.310	4.326	13.0	13.5	104	103	0.32	0.43
	80:20	4.283	4.263	16.2	17.7	154	129	0.23	0.23
ET8	0:100	4.377	4.369	9.8	11.5	56	38	2.51	1.33
	20:80	4.347	4.340	11.8	13.3	72	51	1.17	0.75
	40:60	4.290	4.282	15.5	17.1	73	54	0.85	0.77
	60:40	4.234	4.240	21.7	23.7	73	53	0.33	0.21
	80:20	4.209	4.203	22.1	25.0	107	61	0.30	0.18
p-value (LSD, *p*=0.05)									
CO_2		0.252		0.070		<0.001(3)		0.182	
Soil		<0.001(0.011)		<0.001(0.7)		<0.001(5)		<0.001(0.51)	
Genotype		<0.001(0.007)		<0.001(0.4)		<0.001(3)		<0.001(0.32)	
CO_2 × Soil		0.113		0.188		0.342		0.749	
CO_2 × Genotype		0.323		0.195		<0.001(4)		0.079	
Soil × Genotype		0.025(0.016)		<0.001(0.9)		<0.001(6)		0.099	
CO_2 × Soil× Genotype		0.213		0.242		0.747		0.400	

Table S2. Root length, root and shoot biomass, root-to-shoot biomass ratio (root/shoot) and root diameter of ES8 and ET8 grown for 25 d in the soils with various levels of Al toxicity under two CO_2 concentrations (aCO_2, 400 µmol mol^{-1} and eCO_2, 800 µmol mol^{-1}). Various levels of Al toxicity ($CaCl_2$-extractable Al concentrations of 4.4, 5.8, 7.8, 11.8, 16.5 mg kg^{-1}, respectively) were created by mixing a Dermosol and a Ferrosol at ratios of 0:100, 20:80, 40:60, 60:40 and 80:20 (Experiment 1)

Genotypes	Soil	Root length (m $plant^{-1}$)		Root biomass (mg $plant^{-1}$)		Shoot biomass (mg $plant^{-1}$)		Root/shoot		Root diameter (µm)	
		aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
	0:100	6.8	7.7	55	60	52	58	1.06	1.05	299	300
	20:80	6.5	5.4	54	50	52	49	1.05	1.02	297	315
ES8	40:60	5.4	4.8	50	50	47	47	1.05	1.04	320	328
	60:40	4.4	3.9	46	42	45	43	1.03	0.98	340	342
	80:20	3.5	2.5	47	34	45	37	1.02	0.94	381	386
	0:100	10.0	10.3	56	62	67	66	0.84	0.94	253	262
	20:80	10.0	10.3	59	64	64	66	0.92	0.97	260	266
ET8	40:60	9.1	9.5	57	62	59	63	0.98	0.99	264	269
	60:40	7.3	8.0	52	60	54	60	0.98	1.00	276	292
	80:20	6.7	6.6	51	56	50	56	1.02	0.99	289	304
p-value (LSD, *p*=0.05)											
CO_2		0.148		0.432		0.632		0.692		0.059	
Soil		<0.001(0.5)		<0.001(4)		<0.001(4)		0.590		<0.001(8)	
Genotype		<0.001(0.3)		<0.001(2)		<0.001(2)		<0.001(0.02)		<0.001(5)	
CO_2 × Soil		0.189		0.170		0.781		0.325		0.527	
CO_2 × Genotype		0.016(0.3)		<0.001(4)		0.041(7)		0.030(0.02)		0.547	
Soil × Genotype		0.082		0.027(5)		0.953		0.011(0.04)		<0.001(12)	
CO_2×Soil×Genotype		0.321		0.246		0.092		0.950		0.117	

Table S3. Root length, root and shoot biomass, root-to-shoot biomass ratio (root/shoot) and root diameter of six wheat genotypes grown for 24 d in the Dermosol (pH = 4.12) with lime (4 g $CaCO_3$ kg^{-1} soil) under two CO_2 concentrations (aCO_2, 400 µmol mol^{-1} and eCO_2, 800 µmol mol^{-1}) (Experiment 2)

Genotypes	Root length (m $plant^{-1}$)		Root biomass (mg $plant^{-1}$)		Shoot biomass (mg $plant^{-1}$)		Root/shoot		Root diameter (µm)	
	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
ES8	9.0	10.3	62	75	53	60	1.18	1.26	282	294
ET8	9.8	10.7	72	81	60	64	1.20	1.28	292	299
Egret	10.5	11.7	71	83	57	67	1.23	1.24	268	279
Egret *TaMATE1B*	10.7	11.9	72	82	60	64	1.20	1.28	275	277
EGA-Burke	11.7	13.4	87	100	83	92	1.05	1.09	286	284
EGA-Burke *TaMATE1B*	12.4	14.1	97	108	91	102	1.08	1.06	291	286
p-value (LSD, *p*=0.05)										
CO_2	0.056		0.010 (2)		0.137		0.465		0.225	
Genotype	<0.001 (0.8)		<0.001 (7)		<0.001 (5)		<0.001 (0.07)		<0.001 (10)	
CO_2 × Genotype	0.900		0.988		0.576		0.636		0.503	

Table S4. Concentrations of Al, Mn and Zn in shoots of six wheat genotypes grown for 24 d from sowing in the Dermosol (pH = 4.12) with lime (4 g $CaCO_3$ kg^{-1} soil) under two CO_2 concentrations (aCO_2, 400 µmol mol^{-1} and eCO_2, 800 µmol mol^{-1}) (Experiment 2)

Genotypes	Al (µg g^{-1})		Mn (µg g^{-1})		Zn (µg g^{-1})	
	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2
ES8	64	45	118	135	33.6	32.5
ET8	80	54	136	133	33.2	32.0
Egret	69	46	142	149	31.2	31.3
Egret *TaMATE1B*	50	39	154	150	36.8	34.3
EGA-Burke	59	31	125	118	37.6	29.0
EGA-Burke *TaMATE1B*	41	47	134	132	35.7	34.9
p-value (LSD, *p*=0.05)						
CO_2	<0.001 (7)		0.794		0.018 (1.9)	
Genotype	0.005 (12)		0.011 (16)		0.099	
CO_2 × Genotype	0.190		0.585		0.119	

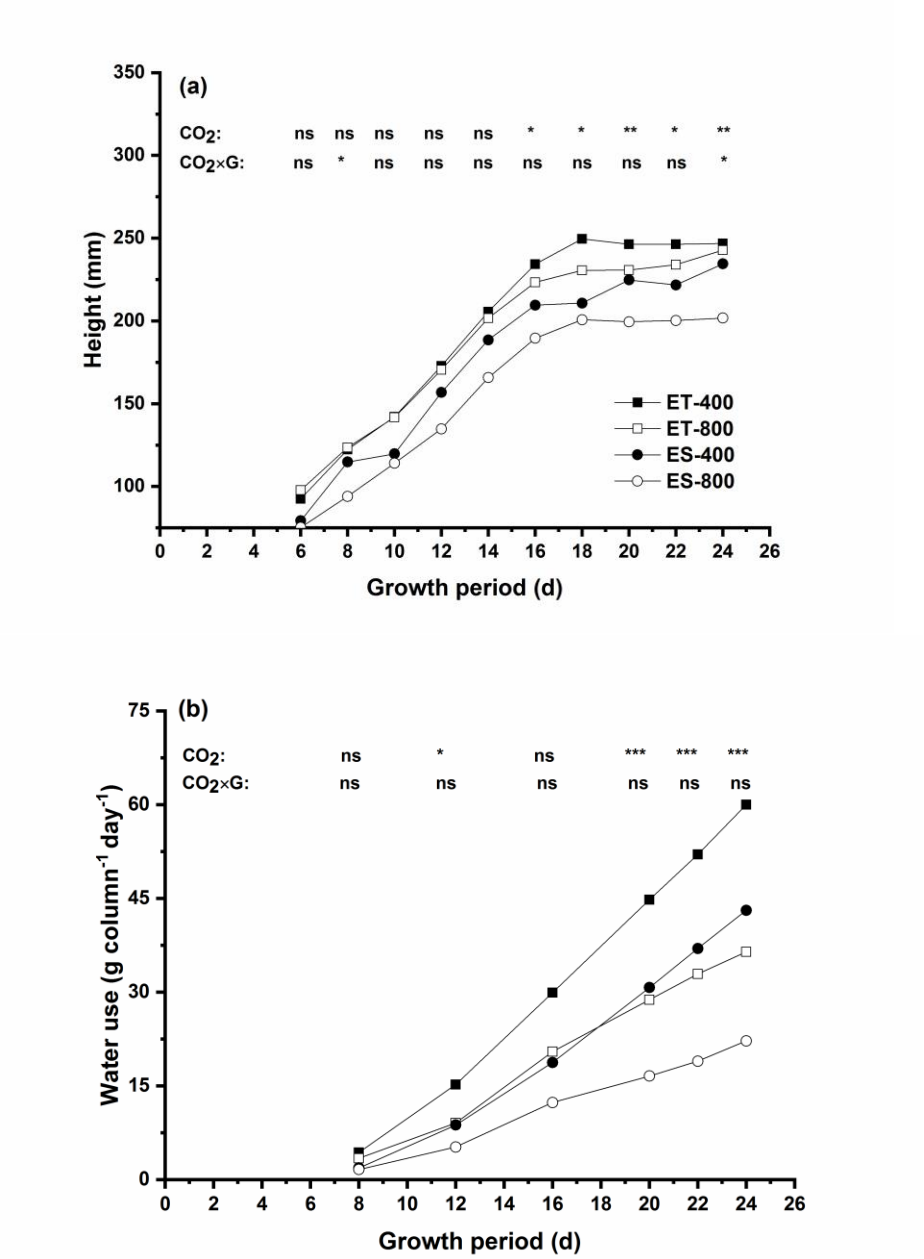


Figure S1. Plant height (a) and water use (b) of ES8 and ET8 grown for 25 d in the most toxic soil (mixture of Dermosol and Ferrosol at a ratio of 80:20) under two CO_2 concentrations (aCO_2, 400 µmol mol^{-1} and eCO_2, 800 µmol mol^{-1}) (Experiment 1). ns, *, **, and *** indicate the significant level at $p>0.05$, $p<0.05$, $p<0.01$, and $p<0.001$ for main effect of CO_2 and interaction of CO_2 and genotype (C