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Translocation and storage of chloride in chlorine-stressed maize (*Zea mays* L.)

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List of Figures

List of Acronyms

ABA	Abscisic acid
A_N	Photosynthesis rate
DM	Dry matter
E	Transpiration rate
ECe	Electrical conductivity of saturated pasta extract (soil)
FM	Fresh matter
g_s	Stomatal conductance
H_2O_2	hydrogen peroxide
NR	Nitrate reductase
NRA	Nitrate reductase activity
NUE	Nitrogen use efficiency
O_2^-	Superoxide radicals
OH	hydroxyl radicals
PGPR	Plant growth-promoting rhizobacteria
ROS	Reactive oxygen species
NAXT1	nitrate excretion transporter 1

1. Summary-Zusammenfassung

1.1 Summary

Maize (*Zea mays* L.) is a moderately salt-sensitive species, its sensitivity to NaCl being mainly associated with the accretion of toxic sodium in shoots for example leading to the sodium-induced damage of leaf chloroplasts. However, less attention has been paid to the effects of chloride (Cl^-). The work described in this dissertation therefore aims at elucidating the physiological adaptations of maize plants to Cl^- salinity. It involves four research questions: 1) how do sensitive maize plants respond to Cl^- salinity with regard to crop yield and plant performance; 2) how are the translocation and tissue storage patterns of Cl^- correlated with tolerance to Cl^- salinity; 3) how do osmotic stress and Cl^- stress impact biomass, chlorophyll content, and nitrate reductase activity (NRA); 4) does sensitivity to Cl^- salinity differ between maize and faba bean plants?

Soil pot experiments and hydroponic culture experiments in the greenhouse have shown that maize is able to withstand Cl^- salinity by being a shoot excluder. The relevant genotypic difference is believed to be based on its ability to undertake Cl^- root-to-shoot translocation. The resistance mechanism of the genotype ES-metronom, which is a more Cl^--tolerant variety, has been attributed to its more efficient shoot exclusion of Cl^-, whereas that of the genotype P8589, which is a more Cl^-- sensitive variety has been ascribed to the preferable sequestration of Cl^- away from the young photosynthetic tissues, such as into old leaf blades, and Cl^- movement in roots possibly to achieve Cl^- dilution. In the mildly tolerant genotype LG30215, osmotic stress does not interfere with NRA but slows down mass flow, which probably reduces NO_3^- transport to leaf tissues, whereas excess Cl^- indirectly inhibits NRA through the antagonistic limitation of NO_3^- uptake. In comparison with maize, faba bean plants are more sensitive to Cl^- salinity rather than to sodium toxicity.

1.2 Zusammenfassung

Mais (*Zea mays* L.) ist eine moderat salzempfindliche Spezies, deren NaCl-Sensitivität vorwiegend mit der Anreicherung von Natrium im Spross verbunden ist. Diese Anreicherung führt unter anderem zu einer Schädigung der Blattchloroplasten. Bisher wurde den Auswirkungen von Cl^- jedoch weniger Aufmerksamkeit geschenkt. Deshalb ist das Ziel dieser Arbeit, die physiologischen Anpassungen von Maispflanzen an Cl^--Überschuss aufzuklären. Dazu werden vier Fragestellungen verfolgt: 1) wie reagieren sensitive Maispflanzen auf Cl^--Salinität im Hinblick auf Physiologie und Ertrag? 2) Korreliert die Translokation und das Einlagerungs-Muster im Pflanzengewebe mit der Toleranz gegenüber Cl^-? 3) Wie wirkt sich osmotischer Stress und Cl^- Stress auf die Biomasse, den Chlorophyllgehalt und die Nitratreduktaseaktivität (NRA) aus? 4) Wie unterscheiden sich Mais und Ackerbohne in ihrer Cl^--Sensitivität?

Gewächshausversuche in Boden und Hydrokultur zeigen, dass Mais der Cl^--Salinität standhalten konnte, da Mais die Verlagerung von Cl^- in den Spross weitgehend unterdrückt. Der genotypische Unterschied hängt vermutlich mit der Wurzel-zu-Spross Translokation von Cl^- zusammen. Die Resistenz im moderat Cl^--toleranten Genotypen ES-Metronom war auf eine effizientere Vermeidung der Verlagerung von Cl^- in den Spross zurückzuführen, während der moderat Cl^--sensitive Genotyp P8589 Cl^- vorwiegend außerhalb von jungen, fotosynthetisch aktiven Blättern, wie zum Beispiel den alten Blattspreiten einlagerte oder in die Wurzeln verlagerte, um einen Cl^--Verdünnungseffekt zu erzielen. In dem moderat Cl^--toleranten Genotypen LG30215 trat durch osmotischen Stress keine Hemmung der NRA, aber eine Verlangsamung des Massenflusses auf, welcher vermutlich den NO_3^--Transport zu den Blättern reduzierte, während Cl^- eine indirekte Hemmung der NRA durch die antagonistische Limitierung der NO_3^--Aufnahme verursachte. Im Vergleich zu Mais ist die Ackerbohne sensitiver gegenüber Cl^--Salinität als zu Natriumsalinität.

2. Introduction

2.1 What is soil salinity?

In recent decades, climate change has seriously impacted the sustainability of the environment, agriculture, and economics (Gruda et al., 2019). The accompanying elevated temperatures attributable to global warming has boosted the burden on arable-land food crops, in particular in arid or semi-arid areas around the world (Wang et al., 2018). Water shortages and the insufficient drainage of irrigated farmlands severely decrease the growth and productivity of agronomic crops (Munns and Gilliham, 2015). Concomitantly, nearly half of all irrigated fields suffer from soil salinity, which reduces water absorption and nutrient uptake (Fita et al., 2015). This leads to osmotic stress, specific ion toxicity, nutrient disequilibrium, and water deficits (Zörb et al., 2019). As a consequence, excess salt ions in plant tissues might disturb photosynthesis and induce chlorosis or even leaf necrosis (Hanin et al., 2016), thereby interfering with crop yields (Zörb et al., 2019).

Salinity is a term used to describe soil conditions with regard to the amount of included soluble salts. Soils are categorized as being saline when the electrical conductivity of saturated pasta extract (ECe) is 4 dS m^{-1} or above (USDA-ARS, 2008), which is equivalent to roughly 40 mM NaCl and which produces an osmotic pressure of around 0.2 MPa. The ECe significantly inhibits the agricultural output of most crop plants (Munns and Tester, 2008). Since the most soluble salt is NaCl, which widely exists in soil, nearly all plants have evolved systems to fight its excess accumulation. In a large proportion of plants, Na^+ and Cl^- are predominantly excluded by roots in order to prevent their entrance into shoots when water is freely being taken up (Munns, 2005a).

2.2 Plant responses to soil salinity

Plant species exhibit great differences in their toleration of salt (NaCl) stress

(Munns and Tester, 2008). In cereals, rice (*Oryza sativa*) is the most sensitive, and barley (*Hordeum vulgare*) is the most tolerant (Islam et al., 2019). Bread wheat (*Triticum aestivum*) is moderately resistant (Colmer et al., 2006), but durum wheat (*Triticum turgidum* ssp. *durum*) is less tolerant (Colmer et al., 2006). Tall wheatgrass (*Thinopy-rum ponticum*, syn. *Agropyron elongatum*) is a representative of the halophyte category and can endure a salt level as high as that in seawater (Colmer et al., 2006). Some legumes such as faba bean, soybean, lentil are sensitive, being even more sensitive than rice (Läuchli, 1984).

The resistant performance of the various crop species is attributed to their diverse responses to two phases of salt stress (Munns and Tester, 2008). In the first phase, osmotic stress is a rapid process decreasing new shoot growth, because it undermines the ability of plants to absorb water and then induces growth reduction. The second phase is ionic toxicity, a slower response caused by the accumulation of ions in transpiring leaves. The biphasic model of salt stress (Figure. 1) suggests that the tolerance of crops can be enhanced by increasing their ability to withstand both phases leading to a constant higher shoot growth rate over the entire life cycle (Munns and Tester, 2008).

In most plants, Na^+ is more likely to attain a toxic level prior to that achieved by Cl^-, and thus, many projects have concentrated on Na^+ exclusion and mechanisms to regulate Na^+ transport (Munns and Tester, 2008). However, some species such as soybean, citrus fruit trees, and grapevines seem to suffer more from toxicity to Cl^- (Läuchli, 1984; Storey and Walker, 1998). The evidence indicates that Na^+ is effectively endured in the woody roots and stems, with only little Na^+ reaching the leaves. Therefore, Cl^-, which continuously passes through the lamina area, becomes the more toxic ion under saline conditions (Munns and Tester, 2008).

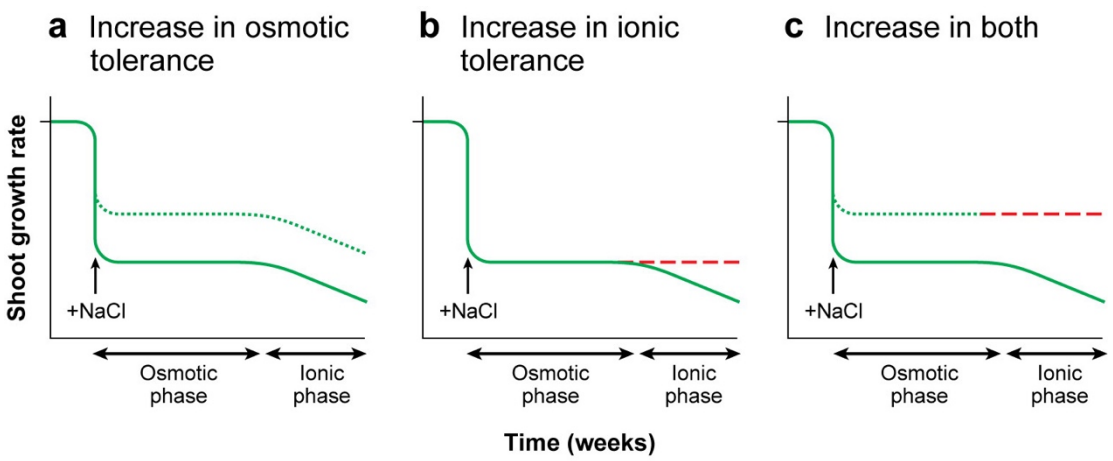


Figure. 1 The growth response to salinity stress occurs in two phases: osmotic stress phase and ion toxicity stress phase (Munns and Tester, 2008). The solid green line represents the change in the growth rate after the addition of NaCl. (a) The broken green line represents the hypothetical response of a plant with an increased tolerance to the osmotic component of salinity stress; (b) The broken red line represents the response of a plant with an increased tolerance to the ionic component of salinity stress; (c) The green-and-red line represents the response of a plant with increased tolerance to both the osmotic and ionic components of salinity stress.

2.3 Adaptive mechanisms of plants to salt stress

Confronted by salinity stress, crops have developed adaptive mechanisms to improve tolerance at both the organ and cellular levels (Figure. 2). In the osmotic phase, the effect dominating the growth response is not specific to salinity stress, but is indeed associated with water deficit. Strategies employed by the plants involve in the improvement of water-use efficiency, osmotic adjustment, the transformation of morphological or developmental patterns to conserve water, and the advancement of the flowering stage (Colmer et al., 2006; Munns, 2005a). Irrespective of the water status, the regulation of phytohormones (such as ABA) (Geilfus et al., 2018) and photosynthate seems to provide the primary management on growth rate under drought or saline stress (Munns, 2005a), because leaf development in plants growing in saline soil is not accompanied with an increase in leaf water relationships within a period of

days (Munns, 2002).

With regard to ion toxicity stress, the first adaptation takes place in the root areas. Roots have to exclude at least 95% of salt ions existing in the soil solution, or the salt has to be delayed from entering into the shoot in order to slow down salt accumulation to toxic ranges (Munns, 2005a). The reason for this is that the transpiration of crops is high, nearly 50 times more water from soil solutions than they retain in shoot tissues (Munns, 2005b). The gate-keepers of the root xylem are located in the parenchyma cells, which can exclude shoot NaCl (Henderson and Gilliham, 2015). Fungus located in root rhizospheres and plant growth-promoting rhizobacteria (PGPR) decreases the Na^+ concentration in shoot tissues, increasingly expresses stress-responsive transcripts, and promotes the synthesis of proline and scavenging of reactive oxygen species (ROS) (De-la-Peña and Loyola-Vargas, 2014; Nadeem et al., 2014). Root architectural systems are of pivotal importance in heightening crop salt tolerance through the changing of root structures to raise their ability to absorb water and nutrients and to restrict salt acquisition (Jung and McCouch, 2013). If a small proportion of salt ions are transported to the shoot, the stem is able either to manage their long-distance transport or to store them immediately in stem tissues (Munns and Gilliham, 2015). In leaf areas, salt tends to be partitioned into the sheath or petiole rather than into the mesophyll, and the ions are re-translocated or excreted (Munns, 2005a; Munns and Gilliham, 2015). Moreover, an alteration of the flowering time and the re-translocation of photosynthate is also advantageous for the improvement of salt tolerance (Colmer et al., 2006; Munns, 2005a). At the cellular level, cells in specific tissues confer the ability to resist the high accumulation of salt ions (Munns and Gilliham, 2015) by osmotic adjustment (Shabala, 2013), cell wall modification, ROS detoxification (Dong et al., 2013; Roy et al., 2014), vesicle trafficking (Bassil and Blumwald, 2014; Garcia de la Garma et al., 2015), transport proteins (Guan et al., 2014), K^+ and NO_3^- homeostasis (Henderson et al., 2014; Shabala, 2013), vacuolar compartmentalization (Garcia de la Garma et al., 2015), and compatible solutes (Shabala, 2013).

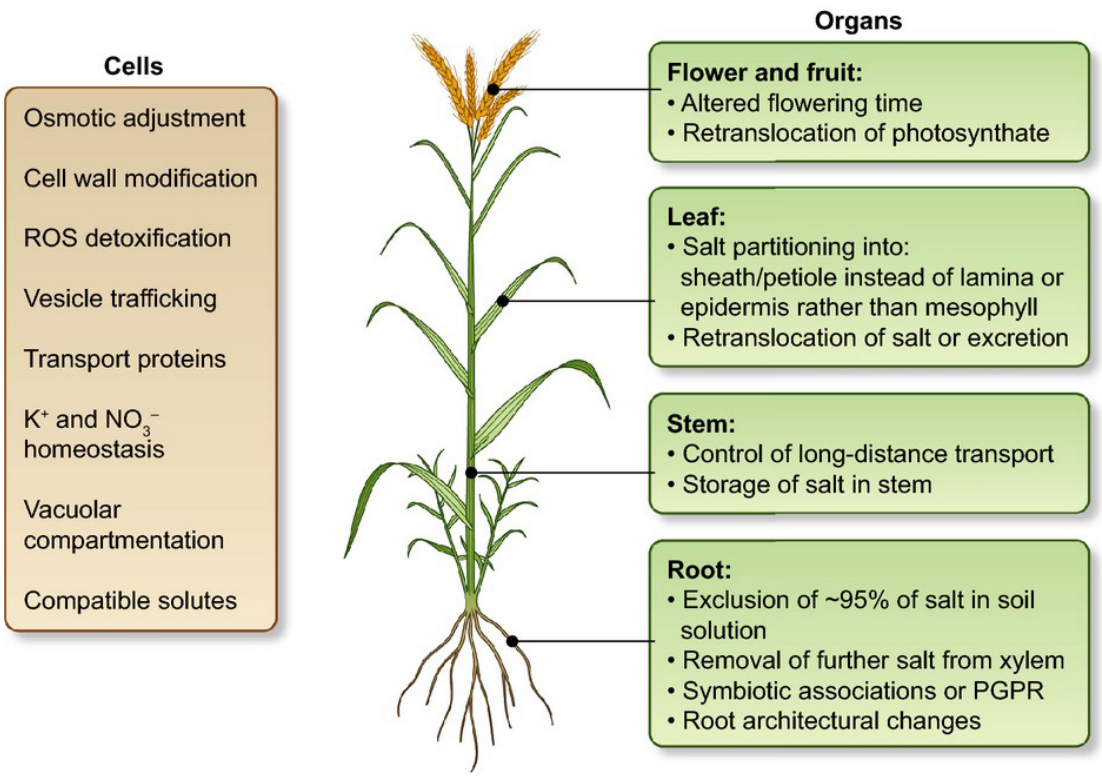


Figure. 2 Adaptive mechanisms of salt tolerance (Munns and Gilliham, 2015). On the left are listed the cellular functions that would apply to all cells within the plant. On the right are the functions of specific tissues or organs. Exclusion of at least 95% (19/20) of salt in the soil solution is needed as plants transpire 20 times more water than they retain (Munns, 2005a). Most of these functions are explained in the text. Omitted for space, and lack of recent advances, is the limitation that Cl^- can impose on growth through its antagonistic accumulation against the nitrogen form nitrate (nitrate homeostasis) (Henderson et al., 2014) and the differential capacity and sensitivity of different cell types and tissues to accumulate Na^+ and Cl^- ; for example, NaCl accumulation within photosynthetic cells incurs a larger cost than accumulation in root cortical cells (Conn and Gilliham, 2010). ROS, reactive oxygen species; PGPR, plant growth-promoting rhizobacteria.

2.4 Chloride: from nutrient to toxicant

Chloride (Cl^-) is the anion form of the halogen chlorine (Geilfus, 2018a) and widely exists in the natural environment (Geilfus, 2018b; Shelke et al., 2019). The

deposition of Cl^- primarily comes from precipitation (0.1 kg of Cl^- ha^{-1} soil DM) (Öberg, 1998), rainwater (11 μM to 8.5 mM) (Xu et al., 1999), sea spray, and rock erosion. The abundance of Cl^- in agricultural land is also attributable to irrigation water (2 to 30 mM) (Xu et al., 1999), dust, air pollution, and fertilizer applications (0.25 mM in untreated plots and 0.73 mM with fertilizers) (Parker et al., 1983). Cl^- is thought to be the anion responsible for the immediate adverse effects of saline water used for crop irrigation (Bar et al., 1997).

Cl^- is required for higher plants as a micronutrient in a μM range (Marschner, 2011). The functional importance of Cl^- involves its wide participation in photosynthesis (Homann, 1987; Kobayashi et al., 2006), especially in the oxygen evolution of photosystem II (PSII) (Kawakami et al., 2009), osmoregulation and turgor regulation (Flowers, 1988), and plant elongation growth (Chen et al., 2016; Franco-Navarro et al., 2015). Besides, Cl^- might be also beneficial for plant growth in the low macronutrient range (mM range) (Franco-Navarro et al., 2015; Franco-Navarro et al., 2019; Raven, 2017). For example, the Cl^- concentration (1-5 mM) has been shown to promote the efficiency of water and nitrogen use in tobacco plants (Franco-Navarro et al., 2015; Rosales et al., 2020). In addition, the function of Cl^- as a micronutrient in photosynthesis, *i.e.*, as a cofactor of the oxygen evolving complex (OEC) during the photosystem II (PSII) (Raven, 2017), has recently been extended as 40 mM Cl^- are required to saturate Cl^- for PSII-OEC assembly / reassembly after photo-damage in *Spinacia oleracea* (Raven, 2020).

In general, the Cl^- concentration in shoot tissues relies heavily on the crop species, cultivar, external environment (White and Broadley, 2001), atmospheric water vapor pressure (Geilfus and Mühling, 2013), and fertilizers containing a Cl^- salt such as KCl (Parker et al., 1983). Minimal Cl^- requirements vary in the shoot tissues of species such as rice (*Oryza sativa*; 3 mg g^{-1} DM), wheat (*Triticum aestivum*; 1.2–4 mg g^{-1} DM), barley (*Hordeum vulgare*; 0.14 mg g^{-1} DM) (Marschner, 2011), spinach (*Spinacia oleracea*, below 0.14 mg g^{-1} DM), lettuce (*Lactuca sativa*, below 0.14 mg g^{-1} DM), and maize (*Zea mays*, 0.05-0.11 mg g^{-1} DM) (Xu et al., 1999). The exception is tobacco (*Nicotiana tabacum*) that has the ability to accumulate to 50 mg g^{-1} DM when

exposed to 5 mM Cl^- (Franco-Navarro et al., 2015). Critical thresholds for Cl^- toxicity in plant tissues have been estimated to be 4–7 mg g^{-1} DM for Cl^--sensitive species and 15–50 mg g^{-1} DM for Cl^--tolerant species (White and Broadley, 2001; Xu et al., 1999).

When salt sensitive species undergo Cl^- salinity, the Cl^- concentration in the plant tissues can easily increase above its required threshold and afterwards can lead to ion toxicity (Geilfus, 2018a). Visual symptoms of Cl^- toxicity usually begin with chlorotic discolorations that gradually turn into necrotic lesions at the leaf tip and leaf edges (Eaton, 1942; Geilfus, 2018a, b; Zhang et al., 2019). Nevertheless, such visual appearances are deceptive and cannot be utilized as an immediate diagnosis of the Cl^- status in specific tissues, because these symptoms are as similar to other nutrient deficiencies (e.g., Cl^- deficiency) or nutrient element toxicities (Geilfus, 2018a). Therefore, a diagnosis of Cl^- toxicity should represent a comprehensive judgement integrating visual symptoms, the absolute Cl^- concentration of tissues, and ion homeostasis.

2.5 Physiological effects of chloride intensity

In soil solution, Cl^- is mobile because of its high water solubility (Reeder, 2006). In preparation to being absorbed, Cl^- is predominantly delivered from the soil medium to the root vascular stele in a symplastic way (Teakle and Tyerman, 2010). Upon being loaded into the root xylem, Cl^- is acropetally transferred to the shoot (Gong et al., 2010), where it is liberated and partitioned by the phloem (Lessani and Marschner, 1978), with most of the Cl^- being stored in cellular vacuoles (De Angeli et al., 2013). Cl^- is believed to increase agricultural productivity in winter wheat (*Triticum aestivum* L.) through elevating the turgor pressure of leaf tissues and consequently accelerating expansion growth (Christensen et al., 1981). It also contributes to the enhancement of water-use efficiency and net photosynthesis rate (A_N) in order to heighten plant development and biomass output in citrus fruit plants (*Cleopatra mandarin*) (Brumos et al., 2010) and tobacco (*Nicotiana tabacum* L. var.

Habana) (Franco-Navarro et al., 2019). Furthermore, it is crucial not only for yield improvement, but also for quality optimization such as protein biosynthesis, blossom-end rot, ethylene production and enhancement of cadmium uptake (Geilfus, 2018b). The osmotic nature of Cl^- can facilitate turgor-driven movements, steer water flow, boost compound migration, and regulate source-sink partitioning (Romo and Haferkamp, 1987).

An excess supply of Cl^- in soil medium facilitates Cl^- uptake and then leads to the elevation of cellular Cl^- to toxic levels, impeding plant growth and development (Geilfus, 2018a). Such developmental suppression is believed to be caused by nutrient imbalance and ion toxicity (Chen et al., 2007; Wu et al., 2013). Imbalanced nutrient status attributable to Cl^- abundance contributes to the enhancement of lipid peroxidation, cellular membrane damage, and the yield of ROS such as singlet oxygen, superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) (Shelke et al., 2019; Wang et al., 2003). An increase of the Cl^- concentration in leaf tissues is harmful as it interferes with the photosynthetic capacity by nonstomatal effects and impairs chlorophyll biosynthesis, promoting chlorophyll degradation and decreasing the actual quantum yield of the PSII electron transport system in association with both photochemical quenching and reducing the efficiency of excitation energy capture (Tavakkoli et al., 2010).

Woody perennial plants (*Vitis* sp. *Citrus* sp., *Persea americana*) and legumes (*Glycine max, Vicia faba*) compartmentalize more Cl^- than Na^+ in leaf tissues (Shelke et al., 2019). The excess storage of Cl^- considerably restricts transpiration, photosynthesis, biomass production, and quality and even accelerates programmed death in many plants (Brumos et al., 2010; Fort et al., 2013; Gong et al., 2010; Luo et al., 2005; Moya et al., 2003; Storey and Walker, 1998; Tavakkoli et al., 2010; Teakle and Tyerman, 2010; Tregeagle et al., 2010; Zhen et al., 2014). The loss in biomass yield and chlorophyll content is also accompanied by an increasing malondialdehyde and proline content and electrolyte leakage in leaf cells under Cl^- stress in the Chrysanthemum (Guan et al., 2012). In addition, exposure of roots to Cl^- disturbs gas exchange through indirect long-distance signal regulation that enables leaf apoplastic

pH transiently to alkalize (Geilfus and Mühling, 2014; Geilfus and Mühling, 2011; Geilfus and Muehling, 2012; Geilfus and Mühling, 2013). The resulting alkalization alters the transcript abundance of the abscisic acid (ABA) biosynthetic genes (Geilfus et al., 2018) and then causes the compartmental redistribution of ABA between leaf apoplasts and guard cells (Geilfus et al., 2015), finally leading to stomatal closure in both the faba bean (*Vicia faba*) and maize (*Zea mays*) (Geilfus et al., 2017). The absorbance and compartmentalization of Cl^- in shoot vacuoles confines nitrogen uptake by competing with NO_3^- transporters (Cubero-Font et al., 2016; Glass and Siddiqi, 1985; Qiu et al., 2016).

2.6 Plants strategic aspects in adaptation to chloride stress

The ability to load and unload Cl^- to the root xylem is the rate-limiting process that decides the amount of Cl^- in the shoot organs under salinity (Li et al., 2017b). The Cl^- concentration in the shoot can be maintained in an endurable range by the restriction of xylem-driven transport from the root to shoot (Geilfus, 2018a). One possibility is the increase of Cl^- efflux from the root cells back to the external medium (Li et al., 2017a; Sun et al., 2009), and another is the reduction of xylem loading and the limitation of acropetal transport (Brumos et al., 2010; Li et al., 2016; Teakle et al., 2007).

For instance, the NO_3^- transporter family NPF2.5 is involved in exclusion of Cl^- out of root cells into the soil medium in *Arabidopsis* (Li et al., 2017a). Potential homologs of NPF proteins (VvNRT1.5 and VvNAXT1) in the grapevine have also been reported to facilitate Cl^- efflux from rootstocks (Henderson et al., 2014). A representative of the anion / H^+ co-transporter family stellar-localized NPF2.4 protein mediates the transport of Cl^- to the shoot; this function is influenced by an ABA increase under saline conditions. The ABA-induced inactivation limits the xylem-delivered Cl^- to the shoot (Li et al., 2016). Moreover, the activity loss of NPF7.3 also seems to impede the upward transfer of Cl^- from root to shoot, since NO_3^- dominates quantitatively over Cl^- (Li et al., 2017b). In *Arabidopsis*, two S-type anion channel

proteins SLAH1 and SLAH3 have been demonstrated to regulate the xylem-driven Cl^- root to shoot translocation (Cubero-Font et al., 2016). Cation / Cl^- co-transporters are responsible for the modulation of the Cl^- build-up in shoot tissues under salinity stress in rice (Chen et al., 2016) and in *Arabidopsis* (Li et al., 2017b).

Given that excess Cl^- is transported into the shoot, it has to be removed from expanding cells and from primary regions of photosynthesis (Tavakkoli et al., 2010; Teakle and Tyerman, 2010). This can be achieved by intercellular and intracellular partition (Fricke et al., 1996; Teakle and Tyerman, 2010). The intracellular compartmentalization of Cl^- into the vacuoles of roots (Storey et al., 2003) or leaf tissues is a common means for maintaining cytosolic Cl^- level below toxic thresholds (Britto et al., 2004; Li et al., 2017b). The Cl^- channel transporter AtCLCa is relevant for Cl^- partition and has a high selectivity for NO_3^- over Cl^- (De Angeli et al., 2006; Wege et al., 2010). Another transporter family of Cl^-, the channel AtCLCg, occurs in the vacuolar membrane of mesophyll cells and contributes to Cl^- storage in NaCl-stressed leaf vacuoles (Nguyen et al., 2016). The third family member AtCLCc is localized to the tonoplast of guard cells and possesses a high selectivity for Cl^- over NO_3^-. During stomatal opening, this transporter family is involved with vacuolar Cl^- sequestration in guard cell and as an osmoticum facilitates guard cell swelling (Geiger et al., 2011; Geilfus, 2018a). The protein ALMT9, a member of the aluminum-activated malate transporter protein family, has a widespread distribution and is located at the leaf tonoplast, leaf vasculature (Barbier-Brygoo et al., 2011), endodermis, pericycle, guard cell, and root vasculature (Eisenach and De Angeli, 2017). It maintains cytosolic ion homeostasis (Baetz et al., 2016). Leaf distribution is also a strategy for avoiding an excess accumulation of Cl^- in expanding or actively photosynthetic tissues (Boursier and Läuchli, 1989). Cl^- is reported to be stored in the cells of the epidermis instead of in the mesophyll (James et al., 2006). In sorghum (*Sorghum bicolor*), Cl^- can be sequestered through the phloem at the leaf sheath rather than in photosynthetically active leaf blades (Boursier and Läuchli, 1989).

Nevertheless, the adjustable capability of tissue sequestration or vacuolar storage is restricted if Cl^- is incessantly taken up under serious Cl^- stress (Geilfus, 2018a).

A mechanism to re-translocate toxic ions from shoot tissues back to the root and the rooting medium via the phloem pathway appears to be feasible and effective (Geilfus, 2018a). The phenomenon of Na^+ re-translocation has been observed in many plant species including the mung bean (*Vigna radiata* L. cv. Berken) (Salim, 1988), tomato (*Solanum lycopersicum*) (Olías et al., 2009), rice (*Oryza sativa* L.) (Yeo and Flowers, 1982), and *Arabidopsis thaliana* (Berthomieu et al., 2003). Some transcripts have been reported to be involved in the process of Na^+ exclusion from the plant tissue back to the rooting solution. For example, excess cytoplasmic Na^+ is driven away by the plasma membrane or vacuolar Na^+/H^+ antiporters, which can be energized by the proton gradient generated by the plasma membrane ATPase (Blumwald et al., 2000). The pathway of Salt Overly Sensitive (SOS) signaling transduction is a fundamental mechanism for modulating Na^+ exclusion by roots (Ji et al., 2013). Specifically, the increasing abundance of the SlSOS1 transcript is able to change the Na^+ sequestration pattern of various tissues (leaves, stem and root) of tomato (*Solanum lycopersicum*) (Olías et al., 2009).

Unfortunately, the situation with regard to the ability of plants to re-translocate (Cl^-) from the tissue back into the rooting solution under conditions of Cl^--salinity is unclear. Although a leaf-brushing experiment with traced ^{36}Cl has shown that Cl^- re-translocation exists and functions better in maize plants than in other species (Lessani and Marschner, 1978), the efflux of ^{36}Cl into the rooting solution was too low (below 3.5%) to relieve the stress on the plant. Moreover, both the tested maize genotypes were considered as being tolerant to a 100 mM NaCl stress, and these plants were concomitantly suffering from Na^+ toxicity. Therefore, we need to test whether Cl^- re-translocation actually takes place in contrasting maize genotypes under Cl^- salinity (excluding the toxic effects of sodium).

2.7 Interaction between chloride and nitrate

As a primary macronutrient, the monovalent anion NO_3^- exhibits a physical property similar to that of Cl^- (Wege et al., 2017). This trait causes an antagonism of

the nutrient uptake between the two anions. The mechanistic explanation for this competition is that both anions, namely Cl^- and NO_3^-, are taken up partly by the same set of transporters (Li et al., 2017b). For example, in the roots of *Arabidopsis*, nitrate excretion transporter 1 (NAXT1) is able to transport Cl^- and NO_3^- but has a higher affinity for NO_3^-. Even when external Cl^- conditions reach 50 mM, the ability to transport NO_3^- is not undermined (Segonzac et al., 2007). In the guard cells of *Arabidopsis*, AtSLAH3 is expressed in the pericycle and is also much more selective for NO_3^- over Cl^- (Li et al., 2017b). By contrast, in maize plants, NPF transporters (Zm-NPF6.6 and Zm-NPF6.4) can transfer either Cl^- or NO_3^-, but Zm-NPF6.4 exhibits Cl^- selectivity, whereas Zm-NPF6.6 is selective for NO_3^- (Wen et al., 2017). When the external Cl^- concentration increases from 0 to 10 mM, Zm-NPF6.4 switches to a high-affinity chloride-selective transporter and significantly reduces NO_3^- uptake, but the selectivity of Zm-NPF6.6 for NO_3^- is little effected. Conversely, the external NO_3^- concentration (from 0 to 1 mM) considerably impacts on Cl^- uptake by Zm-NPF6.6 but marginally influences that by Zm-NPF6.4 (Wen et al., 2017). Nevertheless, these two anions work in coordination to facilitate plant growth. The dual application of both anions substantially enhances nitrogen use efficiency (NUE), photosynthesis, and plant growth in tobacco (*Nicotiana tobaccum*) (Rosales et al., 2020). The beneficial scenario is attributed to cell expansion driven by Cl^- and increased number of plant cells per area by the elevation of NO_3^- availability (Franco-Navarro et al., 2015).

Nitrate reductase (NR) is typically an enzyme induced by the substrate NO_3^-, converting NO_3^- to NO_2^-. A positive correlation between the concentration of NO_3^- as a substrate and nitrate reductase activity (NRA) has been well documented (Hütsch et al., 2016; Mengel et al., 1983; Shaner and Boyer, 1976); however, the causal relationship greatly differs in the tested tissues (Mengel et al., 1983). For example, the correlation of NO_3^- and NRA exhibits saturation in maize leaf but a sigmoidal curve in maize roots. Therefore, N metabolism catalyzed by NR is inevitably modulated by NO_3^- availability. Adverse stresses decrease NRA such as in water shortage (Larsson, 1992; Larsson et al., 1989; Munjal et al., 1997) and NaCl stress (Botella et al., 1993;

Khan et al., 1995; Martinez and Cerda, 1989; Rao and Gnanam, 1990). Nevertheless, these inhibitory effects depend heavily on the severity of abiotic stresses and crop species. The NRA is believed to be relevant for the interference of pertinent RNA expression (Lu et al., 1992) or the transfer of the enzyme to its inactive form (Munjal et al., 1997). Under Cl^- salinity, the excessive uptake of Cl^- can decrease NO_3^- availability and, as a result, disturb NRA. Moreover, Cl^- can also influence the transport of NO_3^- from vacuole to cytoplasm (Aslam et al., 1984) or directly interfere with NR synthesis and its activity (Flores et al., 2000). Nevertheless, the effect of Cl^- intensity on NRA remains unclear in many crops such as maize. This is because, with regard to NaCl salinity, the Na^+ ion has attracted much more attention than the Cl^- ion during the last few decades.

2.8 Research objectives and hypotheses

Maize is renowned as a moderately salt (NaCl)-sensitive crop, its sensitivity to NaCl being associated with the accretion of Na^+ in shoots (Farooq et al., 2015; Shabala et al., 1998). Despite this, Cl^- as a companion ion of Na^+ is rarely paid much attention with regard to its impact on maize plants. The work presented in this dissertation aims at developing a systemic and comprehensive understanding of the physiological responses and the mechanisms providing tolerance to Cl^- stress in maize plants.

Four research questions need to be answered.

(1) How do sensitive maize plants respond to Cl^- salinity with regard to crop yield and plant performance?

(2) How are the translocation and tissue storage patterns of Cl^- correlated with tolerance to Cl^- salinity?

(3) How do osmotic stress and Cl^- stress impact on biomass, chlorophyll content, and nitrate reductase activity?

(4) Do differences occur in the sensitivity to Cl^- salinity between maize and faba bean plants?

3. Publications

The present dissertation is composed of four scientific articles as shown by chapter I-IV and the designed infrastructure is illustrated by technical route (Figure. 3). All individual articles have published.

Publication I

Zhang, X., Zörb, C., Kränzlein, M., Franzisky, B. L., Kaiser, H., Geilfus, C. M. (2019) The early stress response of maize (*Zea mays* L.) to chloride salinity. *Journal of Agronomy and Crop Science*, *205*(6), 586-597. https://doi.org/10.1111/jac.12356

Publication II

Zhang, X., Zörb, C., & Geilfus, C.M. (2020). The root as a sink for chloride under chloride-salinity. Plant Physiology and Biochemistry, 155: 161-168. https://doi.org/10.1016/j.plaphy.2020.06.036

Publication III

Zhang, X., Franzisky, B.L., Eigner, L., Geilfus, C.M., & Zörb, C. (2021) Antagonism of chloride and nitrate inhibits nitrate reductase activity in chloride-stressed maize. Plant Growth Regulation. https://doi.org/10.1007/s10725-020-00685-2

Publication IV

Franzisky, B. L., Geilfus, C. M., Kränzlein, M., Zhang, X., & Zörb, C. (2019). Shoot chloride translocation as a determinant for NaCl tolerance in *Vicia faba* L. *Journal of plant physiology*, 236, 23-33. https://doi.org/10.1016/j.jplph.2019.02.012

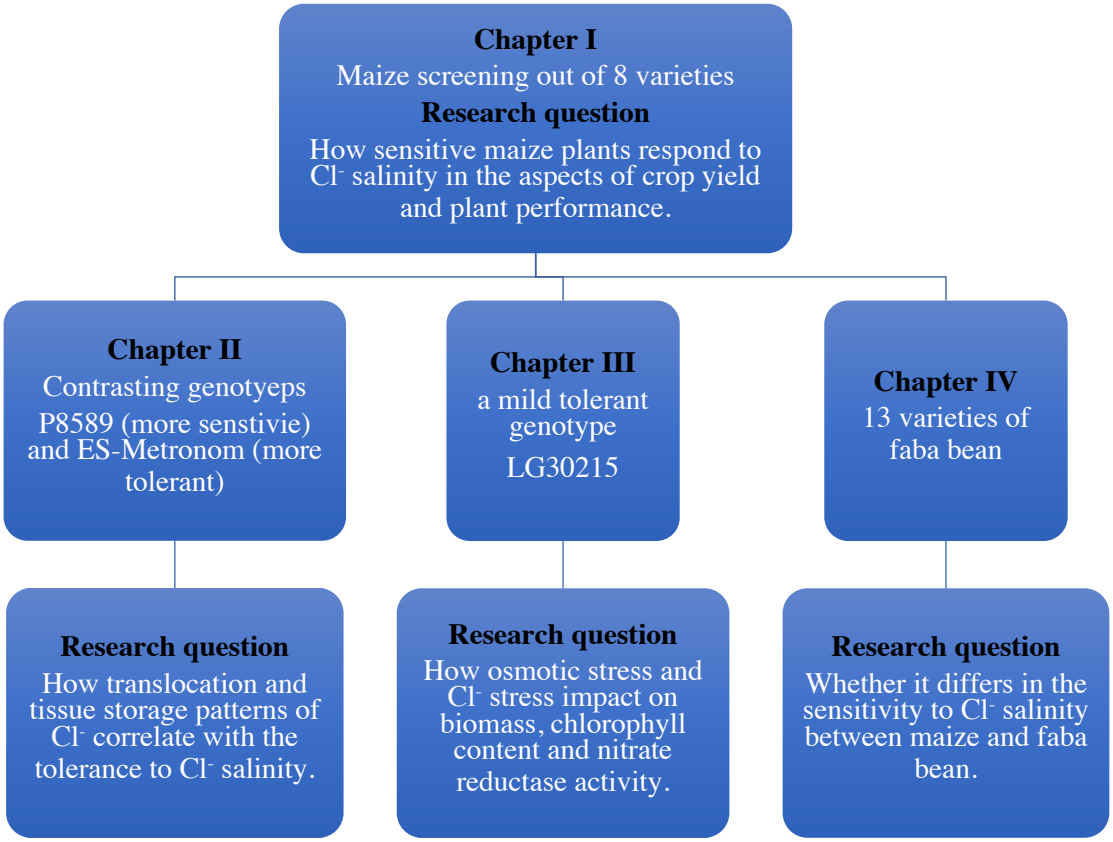
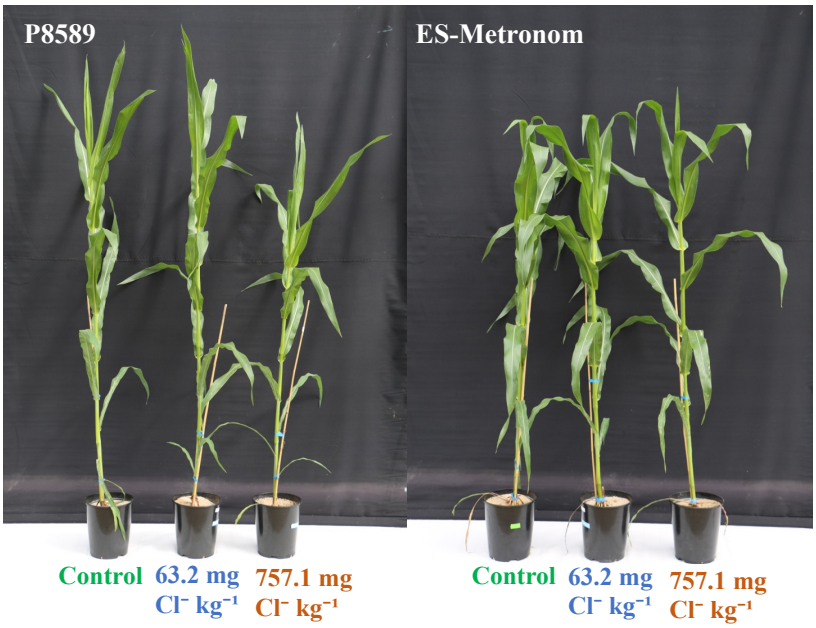


Figure. 3 Technical route

4. Chapter I

The early stress response of maize (*Zea mays* L.) to chloride salinity



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SALINITY STRESS

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The early stress response of maize (*Zea mays* L.) to chloride salinity

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Abstract

Chloride is a micronutrient required for photosynthesis but when applied in the concentration of a macronutrient, it may also promote growth by regulating turgor. However, if chloride accumulates excessively, it can induce toxicity. The aim of this study was to identify physiological dysfunctions in maize (*Zea mays* L.) that arise in response to excessive chloride ion accumulation. For this, a novel water sensor was employed for the first time allowing the in vivo measurement of water content in the plant by using two near IR-wavelengths with different absorption of water. This enabled to analyse whether water imbalances occurred. Chloride was given together with calcium as companying counter cation. Results show that most of the tested maize genotypes were able to maintain growth, photosynthesis and normal water content when stressed with concentrations as high as 757.1 mg chloride/kg soil dry matter. Leaf blades accumulated only 8.5 mg chloride/g dry matter, with the most genotypes not even showing salt stress necrosis at the leaves. A comparison between more tolerant and more sensitive genotypes revealed that restriction of chloride root-to-shoot translocation is a trait of chloride tolerance.

KEYWORDS
chloride salinity, maize (*Zea mays* L.), photosynthetic rate, salt exclusion, tolerance, water content

1 | INTRODUCTION

Chloride (Cl^-) is an element, that is, required for photosynthesis (Arnon & Whatley, 1949). Moreover, it can stimulate the activity of the tonoplast-type H^+-ATPase (Churchill & Sze, 1984; Randall & Sze, 1986) and it can be effective in the regulation of turgor (Fromm & Eschrich, 1989; Geilfus, 2018a). Most glycophytic crop plants contain approximately 1–20 mg/g Cl^- dry matter (DM) (Marschner, 2011). Minimal Cl^- requirements vary in the shoots of crops such as rice (*Oryza sativa;* 3 mg/g DM), wheat (*Triticum aestivum;* 1.2–4 mg/g DM), barley (*Hordeum vulgare;* 0.14 mg/g DM) (Marschner, 2011). In cotton, Cl^- predominantly allocates in vegetative plant tissues such as leaf and stem, which is why the concentration is highest in leaves, being followed by stem, root, seed and fibre (Chen, He, He, Yang, Mishra, & Stoffella, 2010). Maize is regarded as a moderately sensitive crop with regard to NaCl (Farooq, Hussain, Hussain, Wakeel, & Siddique, 2015).

Chloride is the dominant form of chloride in soils. It is very mobile in soil solution because of its high water solubility (Reeder, 2006). For being taken up, Cl^- is transported from the soil solution to the root vascular stele, with symplastic transport as the dominant pathway for Cl^- (Teakle & Tyerman, 2010). Upon entering the root xylem,

Cl^- is acropetally transported to the shoot (Gong et al., 2010), where it is released and may be compartmented by the phloem (Lessani & Marschner, 1978). Cl^- can also be stored in cell vacuoles (De Angeli, Zhang, Zhang, Meyer, & Martinoia, 2013).

Chloride was thought to improve yield in winter wheat by increasing turgor pressure of leaves, facilitating expanding growth (Christensen, Taylor, Taylor, Jackson, & Mitchell, 1981). Generally, Cl^- content is critical not only for yield but also for quality (Geilfus, 2018a). The osmotic properties of Cl^- can increase turgor enabling turgor-driven movements, drive water flow, promoting compound migration and influence source-sink partitioning (Romo & Haferkamp, 1987).

Excessive concentration of Cl^- in soil, as it occurs under NaCl based soil salinity, can lead to an increased Cl^- uptake. As a consequence, cellular Cl^- levels can rise up to toxic levels, hampering plant growth and development (Geilfus, 2018b). High Cl can also alter transcript abundance of abscisic acid (ABA) biosynthetic genes, as shown for maize (Geilfus, Ludwig-Müller, Ludwig-Müller, Bárdos, & Zörb, 2018) and alter compartmental distribution of ABA between the leaf apoplast and the guard cells (Geilfus, Mithöfer, Mithöfer, Ludwig-Müller, Zörb, & Muehling, 2015). The latter controls apoplastic pH (Geilfus, 2017) and stomata closure in salt-stressed field bean (*Vicia faba* L.). A Cl^--induced transient alkalinization of the leaf apoplast stiffens the cell wall during onset of Cl^- salinity in maize leaves, thus being related to the growth reduction under NaCl salinity (Geilfus, Tenhaken, Tenhaken, & Carpentier, 2017).

Excessive Cl^- may accumulate in the chloroplast, which is thought to negatively affect chlorophyll content due to degradation. (Slabu, Zörb, Zörb, Steffens, & Schubert, 2009). As a result, photosynthesis might be impeded giving rise for radical formation. Radicals can destroy photosystem II (PSII) reaction centres (Foyer, Lelandais, Lelandais, & Kunert, 1994). Since Cl^- also acts as an osmoticum, excessive NaCl concentrations in the leaf apoplast may cause cellular damage by disturbing cellular water relations (Oertli, 1968).

Salt exclusion is commonly implemented by preferably accumulating ions in the root or in some relatively insensitive tissues of the shoot of plants under NaCl stress (Boursier, Lynch et al. 1987). Moreover, ion partitioning status of various plant organs is highly related with their potential salt resistance mechanisms. Specifically speaking, a larger amount of Cl^- into the sheath relative to the blade tissue was found in the leaves of young barley (*Hordeurn vulgare L.*) exposed to moderate levels of NaCl salinity (Boursier, Lynch et al. 1987). Cl^- partitioning in the sheaths of plants was also observed in wheat (*Triticum aestivum L.*), maize (*Zea mays L.*) and sorghum (*Sorghum bicolor L.*) (Boursier, Lynch et al. 1984).

This work aimed to screen eight maize genotypes for differences in the ability to grow under conditions of excessive soil Cl^-. A comparison of the individual tissue distribution of Cl^- in those contrasting genotypes was conducted to give clues about the physiological basis of the ability to withstand high Cl^- concentration in the soil, being given as $CaCl_2{}^*2H_2O$. For this, maize were cultivated for nine weeks in Mitscherlich pots using sandy soil. To group the plants into Cl^- includer or excluder, the Cl^- root-to-shoot translocation was determined as the ratio of total shoot Cl^- content to the total root Cl^- content.

2 | MATERIALS AND METHODS

2.1 | Material

Eight maize genotypes (Table 1) were planted (one maize plant per pot) and grown under three Cl^- concentrations (8.75 [control], 63.2 and 757.1 mg Cl^-/kg soil DM, respectively). Chloride was given together with calcium as companying counter cation, using $CaCl_2{}^*2H_2O$. Control conditions equal normal non-saline soils. For the easy sake of reading, we abbreviated the three Cl^- concentrations with "control," "low" and "high" treatment, because Chen et al., (2010) previously reported that maize is a crop with high Cl^--endurance, which was able to tolerate more than 600 mg/kg dry soil without apparent disadvantageous effects. Plants were cultivated in a greenhouse for nine weeks using seven litre Mitscherlich pots filled with 7,840 g DM soil mixture. The soil mixture contained subfloor loam soil (C_{org}, 4.0%; Ostfilden, Stuttgart) homogenously mixed with sand of particle size 0–2 mm according to the ratio of 47.5%/47.5% (w/w). Sour turf soil (Baywa, Filderstadt) (pH = 3.7) was then added with 5% (w/w) based on the prepared soil-sand mixture (pH = 7.26) for adjusting the final pH to 7.06. The surface of the pots was covered with additional 600 g sand per pot. Seeds were sown on 9 June 2017, from then they were watered on a regular basis to maintain 70% (w/w) water holding capacity (WHC) of the potted soil. For fertilization, 2 g NH_4NO_3, 5 g KH_2PO_4, 2 g $MgSO_4{}^*7H_2O$ and 0.3 g Fetrilon-combi micronutrient solution (AgNova Technologies Pty Ltd) was given to each pot as liquid fertilizer. Fetrilon-combi contains micronutrients (1.5% boron, 0.6% copper, 4.0% iron, 3.0% manganese, 0.05% molybdenum and 4.0% zinc) and some macronutrients (0.8% magnesium and 1.3% sulphur). $CaCl_2{}^*2H_2O$ was used to treat the maize with Cl^-. The first Cl^- addition took place with either 21.1 or 252.4 mg Cl^-/kg dry soil DM (given as $CaCl_2{}^*2H_2O$), which were stepwise increased by 10.5 or 126.2 mg Cl^-/kg soil DM, respectively, every second day, finally reaching a maximum dose of either 63.2 for the "low"or 757.1 mg Cl^-/kg soil DM for the "high" (0.5 or 6.4 g/pot DM) after 8 days. The control was not enriched with Cl^-. Treated plants and corresponding controls grew 10 days after full stress treatment was set. At harvest, different plant organs were put into fractions: (a) 9th leaf blade, (b) other leaf blades, (c) leaf sheaths, (d) shoot

TABLE 1 The detailed information of eight maize genotypes

Genotypes	Suppliers
P8589	Pioneer Hi-Bred Northern Europe Sales Division GmbH
LG30222	LG c/o Limagrain GmbH
Tokala	Advanta c/o Limagrain GmbH
KWS-Stabil	KWS SAAT SE GmbH
Amamonte	KWS SAAT SE GmbH
P8400	Pioneer Hi-Bred Northern Europe Sales Division GmbH
LG30215	LG c/o Limagrain GmbH
ES-Metronom	Euralis Saaten GmbH

(A)

Plant fractions	Names given
a	9th leaf blade
b	Other leaf blades
c	Leaf sheaths
d	Shoot
e	Root

(B)

Parameters	Plant fractions used for analysis (shown on part C)
Fresh and dry weight	a, b, c, d, e
Water content	a
Chloride concentration	a, b, c, d, e
Osmolarity	a
Ion leakage	a
SPAD, transpiration rate, stomatal conductance and photosynthetic rate	a

(C)

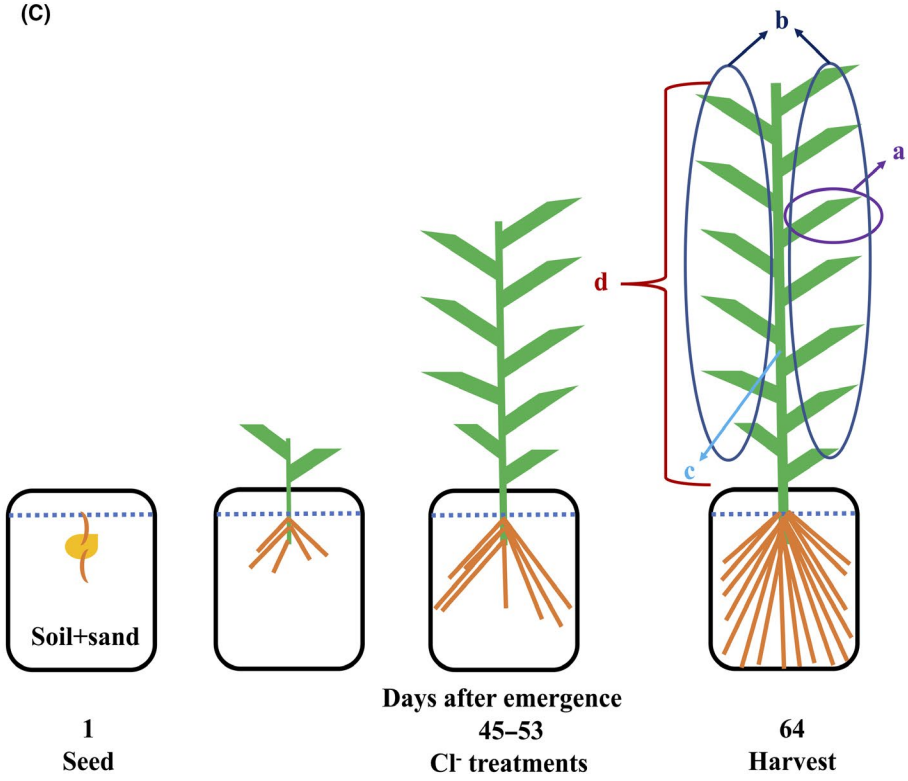

FIGURE 1 Overview of experiment set-up. The first Cl− additions took place with either 21.1 or 252.4 mg Cl−/kg dry soil, which were stepwise increased by 10.5 or 126.2 mg Cl−/kg soil, respectively, every second day, finally reaching a maximum dose of either 63.2 or 757.1 mg Cl−/kg soil (0.5 or 6.4 g/pot) after 8 days. (A) The table describing how plants were harvested into different fractions and how these fractions were named, (B) The table showing which fractions were used for the specific parameter measurements, (C) The schematic chart showing how plants were grown and treated by Cl− and finally harvested

(all leaf blades and leaf sheaths) and (e) root (see Figure 1). It is important to note that fractions (a) and (b) did not contain any leaf sheaths. Each genotype was grown with one plant per pot in five biological replicates. All pots were randomly rearranged twice a week during the whole growth period.

2.2 | Methods

2.2.1 | Fresh weight and dry weight

Fresh weight (FW) of all plant fractions (Figure 1) was weighed immediately after harvest. Dry weight (DW) of plant material was determined after drying at 55°C for 72 hr in a ventilated oven.

2.2.2 | Water content

In order to quantify leaf water content *in planta*, a certain area (diameter = 1 cm) was marked on the 9th leaf blade. On this marked area, we repeated these non-invasive measurements over several days. A water content sensor based on infrared light emitting diodes (LED) and a photodiode linked to a custom device were calibrated against maize leaves and employed for non-invasive measurements of leaf water contents. The schematic diagram of this device was depicted in Figure S1. The underlying principle is the transmission recording of two near IR-wavelengths with different absorption of water penetrating the leaf at an angle of 45°. The ratio of transmission at these two wavelengths is linearly correlated with leaf water content. For calibration of water contents (Table S1), each leaf was completely saturated overnight by floating at 4°C in ddH_2O water, then the water content was determined at six time points (0, 10, 20, 30, 45, 60 min) after the removal from the water bath during the drying process. NIR-transmission ratio and gravimetrically measured leaf water content were determined simultaneously, yielding a linear calibration curve of leaf water content versus NIR-ratio specific for maize leaves.

2.2.3 | Cl^- measurement

Samples from all plant fractions and soils were homogeneously grounded to powder using a mill (Retsch ZM1) equipped with a 0.5 mm sieve. Plant tissues powder (200 mg on a dry basis) and soil powder (2 g on a dry basis) were subjected to Cl^- extraction by solving in 10 ml ddH_2O in glass tubes and heating in water bath at 80°C for 15 min. The suspension was cooled on ice for 7 min and finally filtered through a circular filter paper (90-mm diameter) into a 15 ml falcon tube. Cl^--concentration in the water extract was measured using a Cl^- metre 6610 (Eppendorf) (Ebert, Eberle, Eberle, Ali-Dinar, & Lüdders, 2002). For this, 600 µl of the water extract was mixed with 1 ml gelatin solution (Biorapid GmbH) and 15 ml acid buffer. The stock acid buffer (1 L) was prepared with 0.64% (v/v) nitric acid and 5.76% (v/v) acetic acid (100%). Four technical replicates were conducted.

2.2.4 | Osmolarity

The leaf sap was collected at 10 days after full stress treatment by squeezing the 9th leaf blade of each plant. Leaf sap was stored at −20°C. Osmolarity was measured with semi-micro osmometer (Knauer ML, Berlin, Germany) (Zimmermann et al., 2008) by diluting the original sap 1:4 using ddH_2O water. Aliquots of 200 µl were used for determination. A standard curve was made by 10, 20, 30 and 40 mM $CaCl_2$*$2H_2O$ for calculating the osmolarity in leaf sap. Each measurement was conducted in replicates of four.

2.2.5 | Leaf electrolyte leakage

When harvesting the plant material, six 1 cm diameter discs were immediately collected from the 9th leaf blade and washed for four times with ddH_2O. Then, they were put into a 50 ml falcon tube containing 20 ml ddH_2O. The conductivity was firstly measured after 4 hr of shaking using a conductometer (WTW LF90 and a WTW KLE1 cell, Weilheim, Germany) (El Achouri et al., 2001). The leaf discs were stored overnight at −20°C, and then the total conductivity was recorded after thawing. Ion leakage was expressed as the ratio of the conductivity (4 hr) and the total conductivity.

2.2.6 | Electrical conductance and pH of soil solution

After harvest, electrical conductivity of potted soil solution was directly measured by a handheld readout device (Infield 7) (De Neve et al., 2018) equipped with Theta Probe (ML2x). For determination of soil pH, 2 g dry soil powder with a particle size of 0.5 mm was dissolved in 25 ml ddH_2O and incubated on a shaker for 45 min. After 15 min sedimentation, the pH in the supernatant was measured using a pH meter (WTW 538) (Ullrich, Menge, Menge, Schmid, Gübitz, & Krauss, 2001).

2.2.7 | Transpiration rate, stomatal conductance and photosynthetic rate

The area that was marked in the 9th leaf blade (Figure 1) for water content quantification (see section 2.2.22) was also used to monitor photosynthetic rate and transpiration rate using a LCi Portable Photosynthesis System (ADC BioScientific Ltd) (Ramani et al., 2006). A broad type chamber was used, the observed leaf area was 625 mm^2 and the light was natural sunlight lying between 0.4 and 3.0 microns. For maize leaves, CO_2 flowing into leaf chamber was around 400 vpm and H_2O flux was between 0.17 µmol/m^2 s^{-1}.

2.2.8 | Chlorophyll concentration

The chlorophyll concentration in the 9th leaf blade was measured by a Chlorophyll meter (SPAD 502; Konica Minolta) (Netto, Campostrini, Campostrini, Oliveira, & Bressan-Smith, 2005). For each leaf, four measurements were conducted at different positions on opposite sides of the central vein.

2.3 | Statistical analysis

All data were expressed as mean ± standard error. To determine the significance difference between control and two levels of treatments, data were analysed by Duncan test (three and more variables) or *T* test (two variables) at the probability of 0.05 and 0.01 levels with SPSS software 19.0 (SPSS Inc), as indicated in the figures. Principal component analysis (PCA) was conducted by SPSS 19.0 on a basis of all parameters measured above. For each sample, every measurement had five biological replicates. Only Cl^- concentration and osmolarity measurements were technically repeated four times while other parameters had no technical replicates.

3 | RESULTS

3.1 | Fresh and dry biomass of different maize organs

Treating maize with either 0.5 (low) or 6.4 (high) g Cl^- per pot did not significantly change FW in the 9th leaf blade, other leaf blades, leaf sheaths and the shoot of almost all genotypes (Figure 2). This pattern was also reflected by DW (Figure 3B). The genotype P8589 was an exception because it developed leaf edge and leaf tip necrosis (Figure S2C) and FW and DW were significantly reduced in the fraction that we call "other leaf blades" (this fraction represents all leaf blades but not the ninth leaf blade) when high Cl^--treatment was applied (Figure 2). P8589 stood out for a second reason: this genotype increased FW and DW under low Cl^- treatment. However, this trend was only significant for FW in the leaf sheaths of P8589 (Figures 2 and 3). Root biomass was not affected by low or high Cl^- treatment in any genotype, except for KWS-Stabil, displaying significantly reduced root DW under high Cl^- (Figure 3E). Other leaf blades had significant lower calculated water content (58.5 g) under high Cl^- treatment in P8589 in comparison with control (72.3 g) (Figure 4B). However, this trend was not observed in leaf sheaths, in which low Cl^- supply increased calculated water content from 212.1 g to 243.8 g in P8589 (Figure 4C). Similarly, P8589 and P8400 exhibited a greater calculated water content (330.2 g and 296.8 g, respectively) in shoot under low Cl^- level than the corresponding controls (291.8 g and 266.9 g, respectively) (Figure 4D).

3.2 | Cl^- distribution in 9th leaf blade, other leaf blades, leaf sheaths, root and soil

In soil, Cl^- concentrations expressed as average over all pots of all genotypes were 8.8 mg Cl^-/kg soil DM in control, 26.4 mg Cl^-/kg soil DM in low treatment and 335.4 mg Cl^-/kg soil DM in high treatment (Figure 5E).

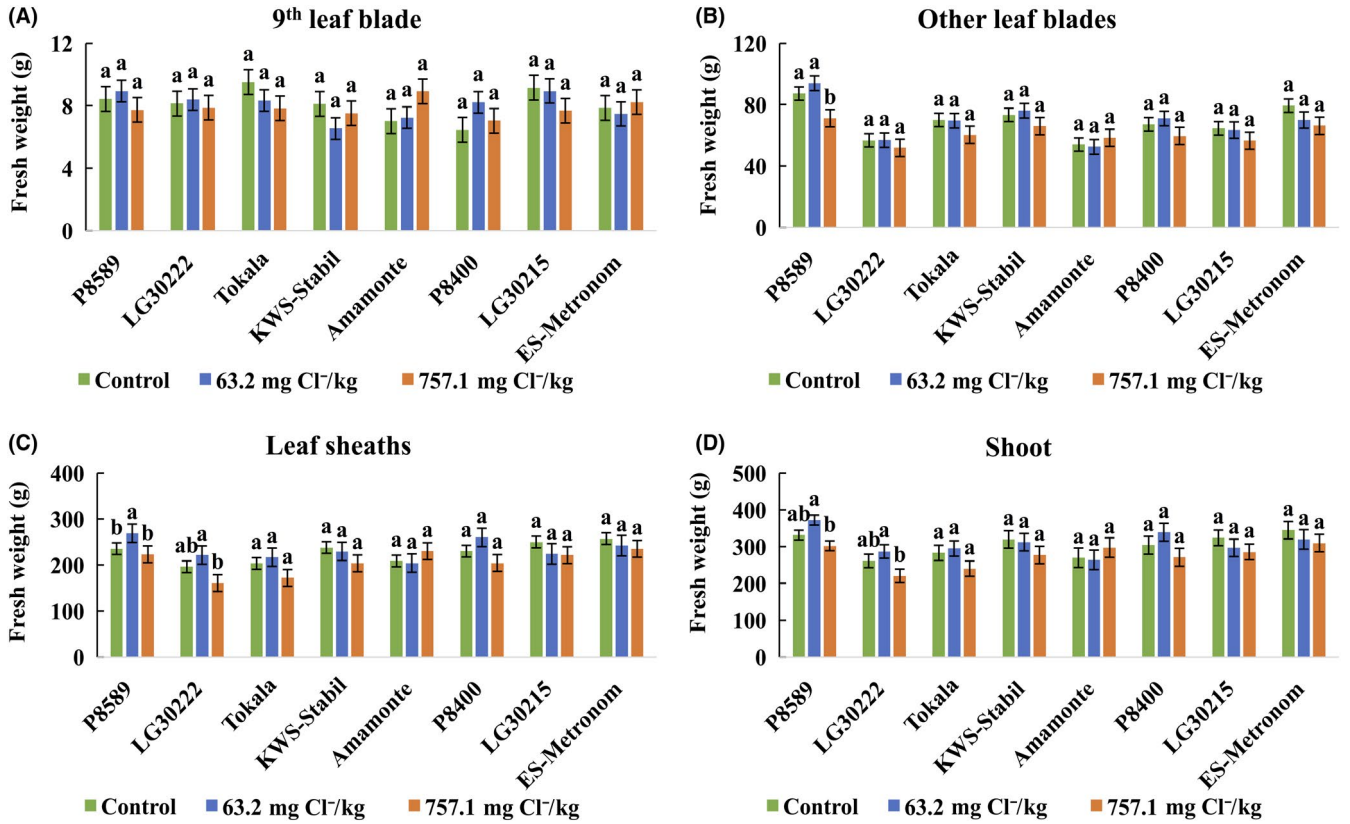

FIGURE 2 Fresh weight in different plant tissues. Small letters indicate significant FW mean difference ($p < 0.05$) under different treatments per genotype by Duncan Test. (A) Fresh weight of 9th leaf blade, (B) Fresh weight of other leaf blades, (C) Fresh weight of leaf sheaths, (D) Fresh weight of shoot

In the 9th leaf blade, other leaf blades, leaf sheaths, roots and soil, Cl^- concentration significantly increased with rising external Cl^- application. This was true for all genotypes except for the root of P8589, the 9th leaf blade of KWS-Stabil and the fraction "other leaf blades" (this fraction represents all leaf blades but not the ninth leaf blade) in ES-Metronom, where no significant increases in Cl^- concentration could be observed after low treatment compared with controls (Figure 5). When plants were stressed by a high Cl^--dose, as expected, all genotypes showed significant Cl^- increase in all plant fractions. Besides, LG30222 had the greatest shoot/root ratio and Amamonte, P8400 and ES-Metronom were the lowest under high Cl^- treatment (Figure 5F). The genotype Tokala showed the same low shoot/root ratio under either low or high treatment (Figure 5F).

3.3 | Water content

In comparison with control, low Cl^- treatment did not change the non-invasively quantified water content of all genotypes (Figure 6). However, as expected, non-invasively quantified water content was significantly reduced from 13.9 mg/cm^2 to 10.8 mg/cm^2 and from 12.7 mg/cm^2 to 10.6 mg/cm^2 in the genotypes Amamonte and P8589, respectively, by high Cl^- treatment. In contrast, genotypes KWS-Stabil and LG30215 had increased non-invasively quantified water content from 11.3 mg/cm^2 to 13.9 mg/cm^2 and from 9.8 mg/cm^2 to 11.6 mg/cm^2, respectively, when exposed to high Cl^- concentration. The non-invasively quantified water content in genotypes LG30222 and ES-Metronom remained unchanged irrespective of the treatment.

3.4 | Osmolarity and electrolyte leakage

Treating maize with either low or high Cl^- increased osmolarity from 258 mOsm/L to approximately 275 mOsm/L in Amamonte and from 277 mOsm/L to approximately 297 mOsm/L in LG30215, respectively, but did not affect osmolarity in Tokala, P8400, LG30222 and ES-Metronom (Figure 7A). Under high Cl^- treatment, the osmolarity of P8589 and KWS-Stabil increased from 245 mOsm/L to 292 mOsm/L and from 237 mOsm/L to 272 mOsm/L, respectively, whereas osmolarity was not affected by low Cl^- treatment in these two genotypes. None of the genotypes showed a significant difference in ion leakage between both Cl^- treatments (Figure 7B).

3.5 | Soil electrical conductance

Under both Cl^- treatments, electrical conductance in the soil kept the same value in comparison with controls among all genotypes (Figure 8). However, the genotypes KWS-Stabil and P8400 were the exceptions. Electrical conductance of soil in pots of KWS-Stabil

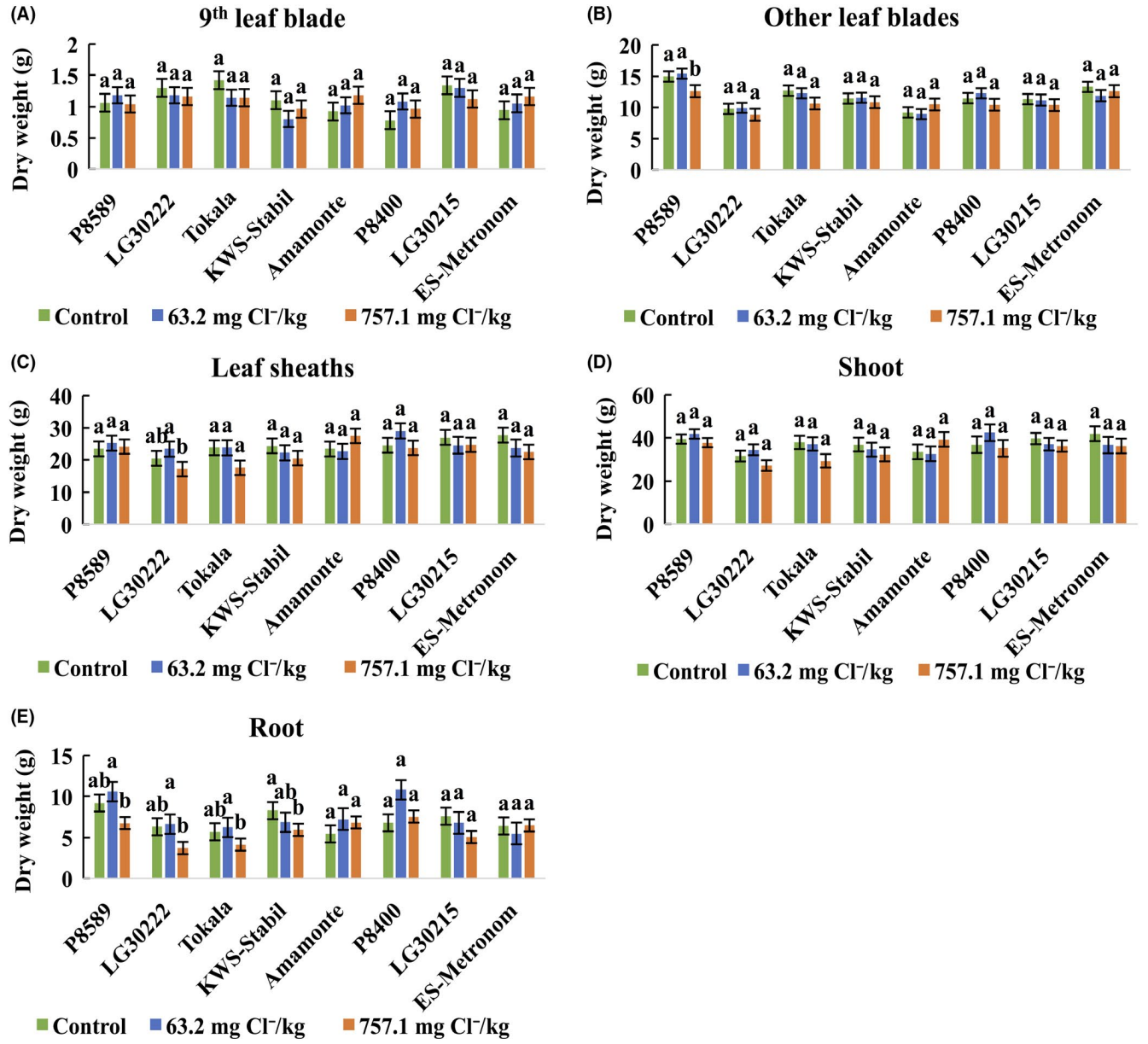

FIGURE 3 Dry weight in different plant tissues. Small letters indicate significant DW mean difference ($p < 0.05$) under different treatments per genotype by Duncan Test. (A) Dry weight of 9th leaf blade, (B) Dry weight of other leaf blades, (C) Dry weight of leaf sheaths, (D) Dry weight of shoot, (E) Dry weight of root

increased under both Cl^- treatments. In contrast, soil electrical conductance of P8400 only increased with high Cl^- addition (Figure 8).

3.6 | Transpiration rate, stomatal conductance, photosynthetic rate and SPAD

Transpiration rate, stomatal conductance and photosynthetic rate of 9th leaf blades of all genotypes were unaltered by both Cl^- treatments during the whole growing period (Figure 9). However, an effect of Cl^- application on chlorophyll concentration estimated by SPAD measurements was found. In comparison with control, chlorophyll concentration of P8589, LG30222 and P8400 was significantly increased by low Cl^-. In contrast, high Cl^- treatment considerably decreased chlorophyll concentration from 50.7 to 49.3 and from 54.7 to 51.4 in genotypes LG30222 and LG30215, respectively.

3.7 | Principal component analysis

Principal component analysis (PCA) revealed that Cl^- concentration was the variable that explained most of the variance on principal component 1 (30.9%), whereas water content was the dominant factor for the variance on principal component 2 (16.3%) (Table S2 and Figure 10). Consequently, the genotypes clustered predominantly into groups being affected by the dose of the Cl^- treatment (Figure 10). However, two genotypes deviated from this trend. Low Cl^--treated P8589 was located in the group of high Cl^- concentration and low Cl^- treated ES-Metronom clustered into control group.

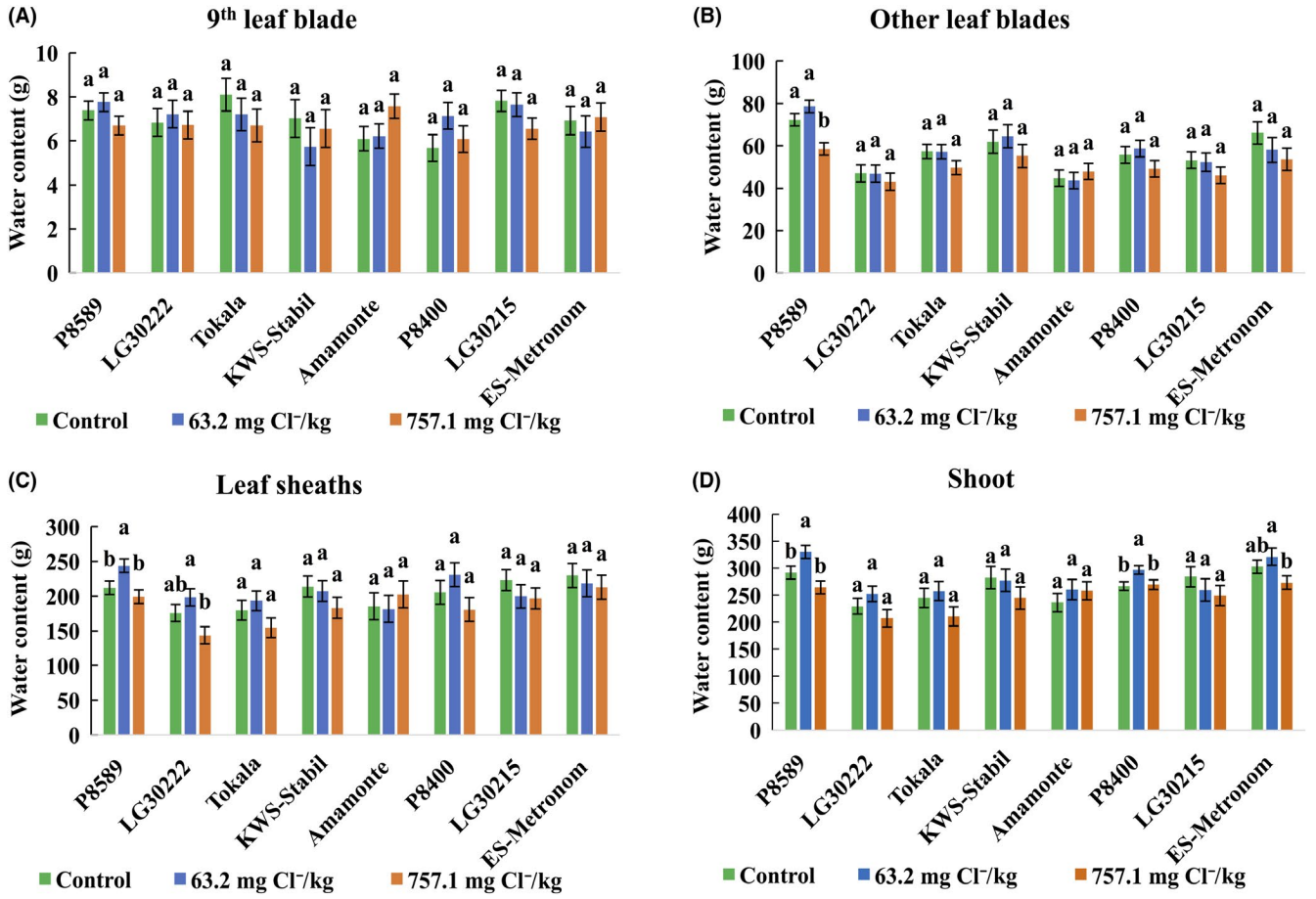

FIGURE 4 Calculated water content of plant fractions. Small letters indicate significant mean difference ($p < 0.05$) under different treatments per genotype by Duncan Test. (A) Water content of 9th leaf blade, (B) Water content of other leaf blades, (C) Water content of leaf sheaths, (D) Water content of shoot

4 | DISCUSSION

4.1 | Treatment and soil condition

In this experiment, we applied calcium as Cl^- accompanying cation because the macronutrient calcium is not toxic at the applied concentration (Kirkby & Pilbeam, 1984; Marschner, 2011). Changing soil calcium concentration may influence soil pH, however, in our experiment soil pH was stable (Table S3). With these prerequisites, it is most likely that the series of physiological reactions and effects described in this work are attributable to different Cl^--treatments. The high amount of Cl^- applied (757.1 mg/kg soil) in the experiment was still mild stress for maize, because neither severe chlorotic nor necrotic lesions could be observed. Only genotype P8589 showed necrosis at the tip and margin of the 9th leaf under low and high treatment, respectively.

4.2 | Effects of chloride salinity in contrasting genotypes

Our data indicate that Cl^- root-to-shoot translocation was restricted in most maize genotypes, with the expectation of P8589, as the shoot-to-root ratio was less than 0.5 (Figure 5F). Thus, we conclude that maize is an Cl^- excluder. However, some Cl^- accumulates in the aerial part of the plant. Twice as much of Cl^- was present in the leaf sheaths as compared to the leaf blades under low and high treatment. This pattern could prevent harmful effects on photosynthesis, as leaf blades are more active in photosynthesis compared to the sheaths.

Although leaf sheaths DW of P8589 was similar under low and high Cl^- (Figure 3C), leaf sheaths FW of low Cl^- treated P8589 plants was higher than that of high Cl^- treatment (Figure 2C). This shows that only high Cl^- treatment induced stress in P8589. This was true for other leaf blades of P8589 as well (Figure 4B). This observation was also verified by sensor-measured water content and osmolarity data on the 9th leaf blade (Figures 6 and 7A) that underlined that high Cl^- treatment caused a reduction in water content, indicating osmotic stress. Furthermore, plant height of P8589 under low Cl^- concentration kept the same as control (Figure S2). However, this phenomenon was not seen under high Cl^- treatment, which indicates that the development of P8589 was reduced when Cl^- reached too high concentrations (Figure S2). Nevertheless, such reduction was probably attributed to the osmotic stress instead of ion toxicity, since the permeability and integrity of cellular membrane in leaf blades were not affected under such high Cl^- treatment (Figure 7B). Overall, the genotype P8589 was identified as being particularly sensitive to Cl^--stress, possibly due to problems in maintaining

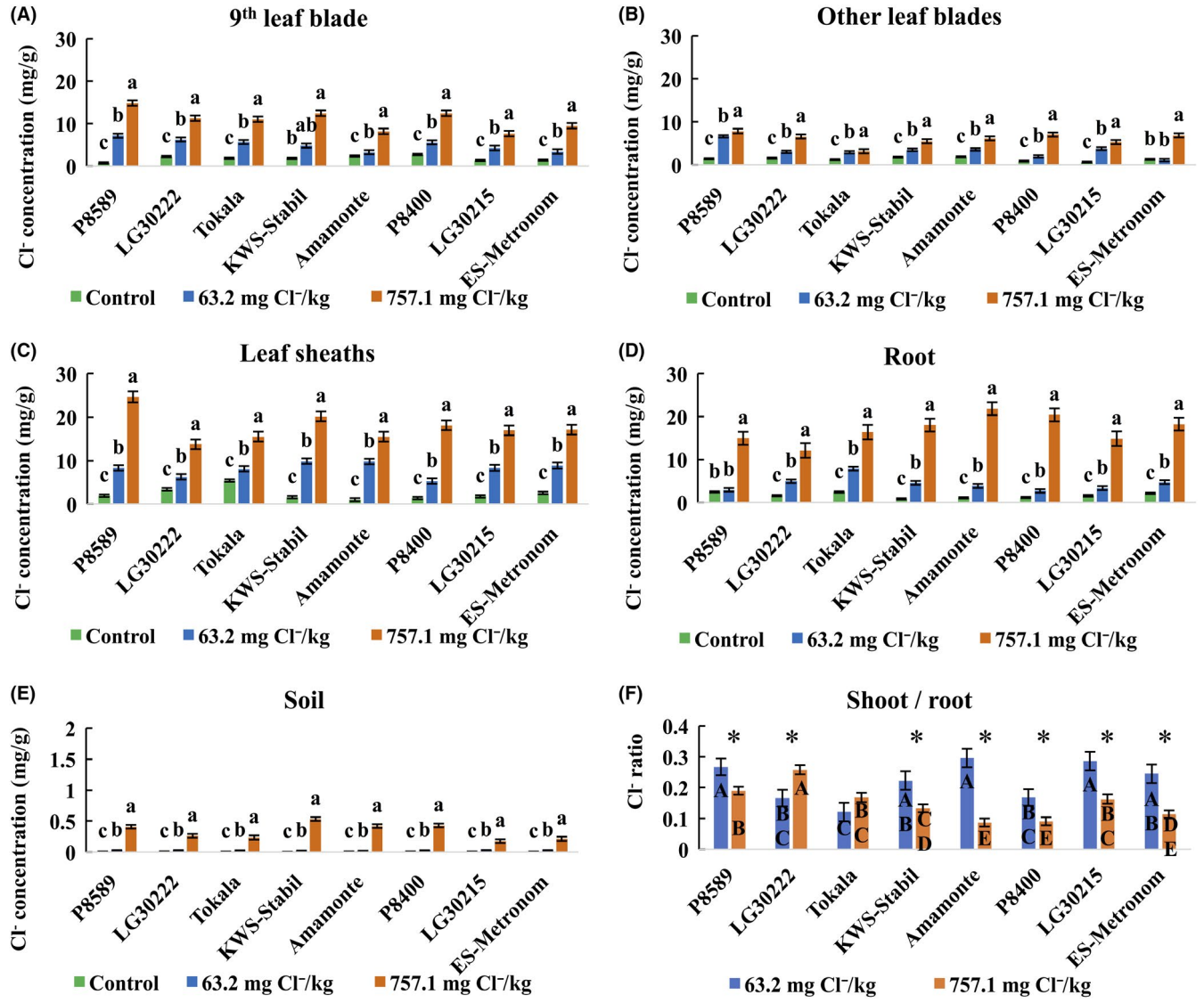

FIGURE 5 Chloride distribution in different plant tissues and soil. Small letters indicate significant mean difference ($p < 0.05$) under different treatments per genotype by Duncan Test; capital letters in shoot/root ratio indicate significant mean difference ($p < 0.05$) among all genotypes under one treatment; * and ** indicate significant mean differences ($p < 0.05$ and $p < 0.01$, respectively) r in shoot/root ratio between 63.15 mg Cl^-/kg and 757.11 mg Cl^-/kg for each genotype by Duncan Test. (A) Cl- concentration of 9th leaf blade, (B) Cl- concentration of other leaf blades, (C) Cl- concentration of leaf sheaths, (D) Cl- concentration of root, (E)Cl- concentration of soil, (F) The ratio of Cl- concentration between shoot and root

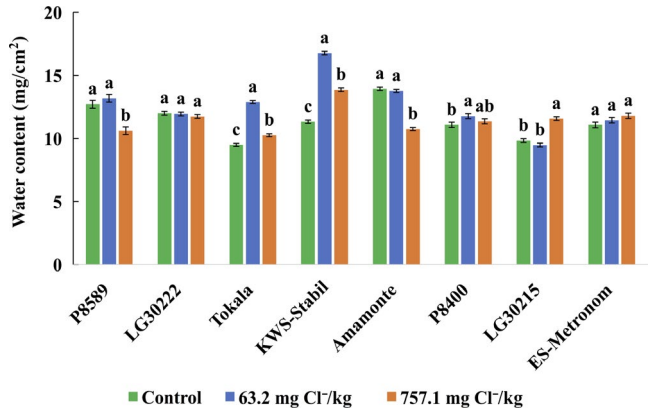

FIGURE 6 Water content in the 9th leaf blade. Small letters indicate mean significant difference in water content ($p < 0.05$) under different treatments per genotype by Duncan Test

normal cellular water relations in plant tissues. Necrotic leaf edges witness chloride toxicity (Figure S2C).

A different tendency was detected in ES-Metronom. Neither low nor high Cl^- treatment influenced biomass formation (FW and DW), osmolarity or water content. Moreover, photosynthetic rate, stomatal conductance and transpiration rate of ES-Metronom were not affected by subjection to low or high Cl^- treatment (Figure 9). This implies that ES-Metronom was more tolerant to Cl^- stress than P8589 as even high Cl^- did not induce osmotic nor ion-toxic stress.

A potential explanation for the different performance under high Cl^- between P8589 and ES-Metronom could be attributed to a differing allocation of excess Cl^- within the plant organs according to shoot/root ratio (Figure 5F). In our findings, ES-Metronom

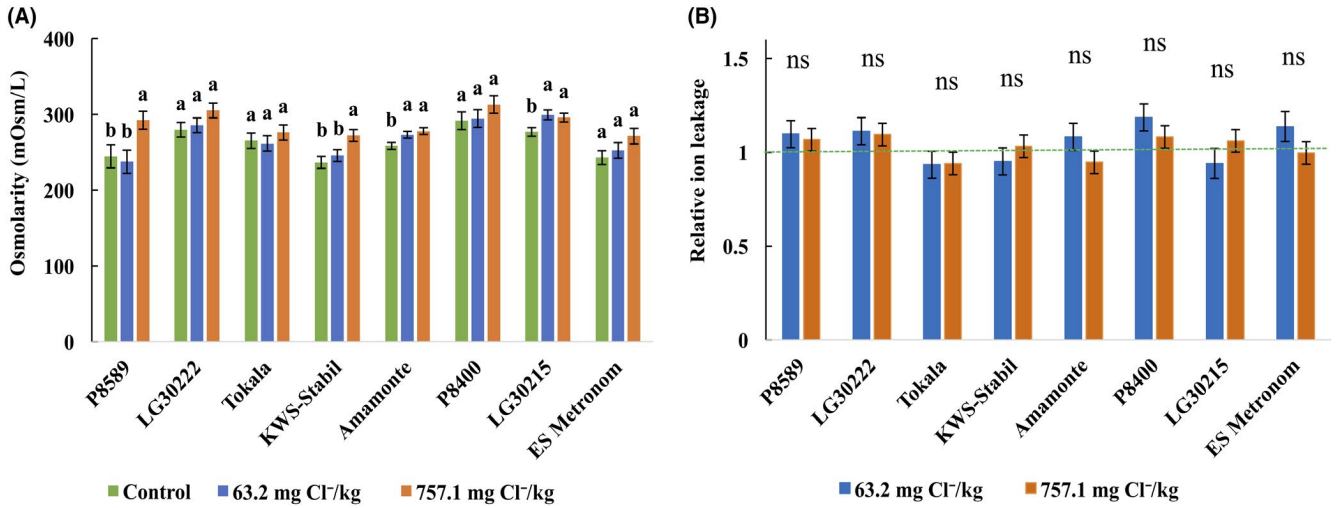

FIGURE 7 The osmolarity and electrolyte leakage in the 9th leaf blade sap. Small letters indicate significant mean difference ($p < 0.05$) in osmolarity under different treatments per genotype by Duncan Test; relative ion leakage was expressed as low treatment/control- and high treatment/ control-ratio; *T* test was used for analysing significant difference in electrolyte leakage. The sign "ns" means non-significant. (A) Osmolality of 9th leaf blade, (B) Relative ion leakage of 9th leaf blade

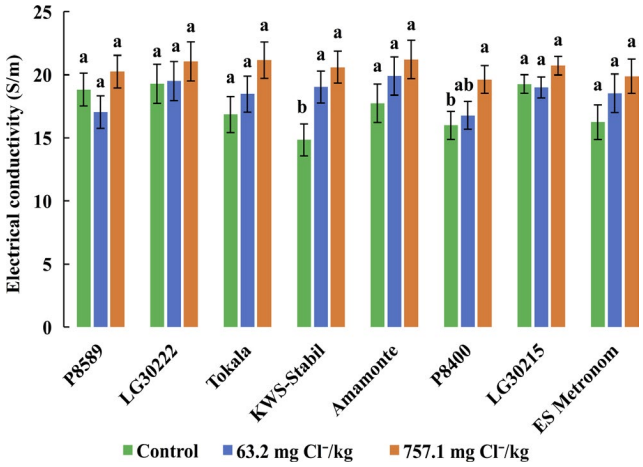

FIGURE 8 Electrical conductance of soil. Small letters indicate significant mean difference ($p < 0.05$) in electrical conductance under different treatments per genotype by Duncan Test

had lower shoot/root ratio under both low and high treatment than P8589. This indicates that ES-Metronom was able to exclude Cl^- from being transported to the shoot. There is a strong correlation between sodium exclusion and salt tolerance in many cop species (Flowers & Yeo, 1986; Munns & James, 2003). Our data indicate this for Cl^-, as previously done by others (Brumos, Talon, Talon, Bouhlal, & J. M. COLMENERO-FLORES, 2010; Li et al., 2016; Teakle, Flowers, Flowers, Real, & Colmer, 2007).

This is useful as ongoing salt ion accumulation will cause osmotic imbalances and ion toxicities (Munns, James, James, & Läuchli, 2006). Therefore, restricting acropetal transport of Cl^- is likely to be an important factor contributing to low salt accumulation in leaves and might be the underlying mechanism of increased Cl^- tolerance of ES-Metronom (Munns, 2005; Pitman, 1984). We speculated that this reduced transport is based on restricted xylem loading, however, we have not tested this. However, Cl^- tolerance by Cl^- retention in root seems to be trade-off as the root growth of the respective genotypes was impaired to some extent, most likely due to the excessive Cl^- accumulation.

4.3 | No relation between osmolarity, Cl^- and growth

Osmolarity of P8589, KWS-Stabil, Amamonte and LG30215 (Figure 7A) was increased by high Cl^- treatment. Apparently, Cl^- accumulated in the cells, acted as osmoticum and facilitated water uptake. Growth, however, was not facilitated as it might have been possible in the light of the turgor-driven acid-growth. Shoot fresh and dry biomass of all maize genotypes were not affected by low or high chloride treatment in comparison with controls (Figures 2D and 3D). Similar results were reported by Hütsch, Keipp, Glaser, and Schubert (2018) who figured out that the potato cultivars Marabel and Désirée can be fertilized with KCl instead of K_2SO_4 without the risk of tuber yield depression.

4.4 | Chlorophyll concentration and photosynthetic rate

The chlorophyll concentration in genotypes LG30222 and LG30215, as estimated by SPAD readings (Figure 9D), was reduced by high Cl^- treatment in comparison with controls. Slabu et al. (2009) reported that a reduced chlorophyll concentration in leaves of *Vicia faba* after NaCl exposure (13 days after 100 mM treatment) is attributable to high chloroplastic Cl^- concentrations rather than the accumulation of sodium. Chloroplasts exhibit a high permeability for Cl^-, and a treatment with NaCl resulted in the accumulation of Cl^- and decline in SPAD values (Heber & Heldt, 1981). Furthermore, high

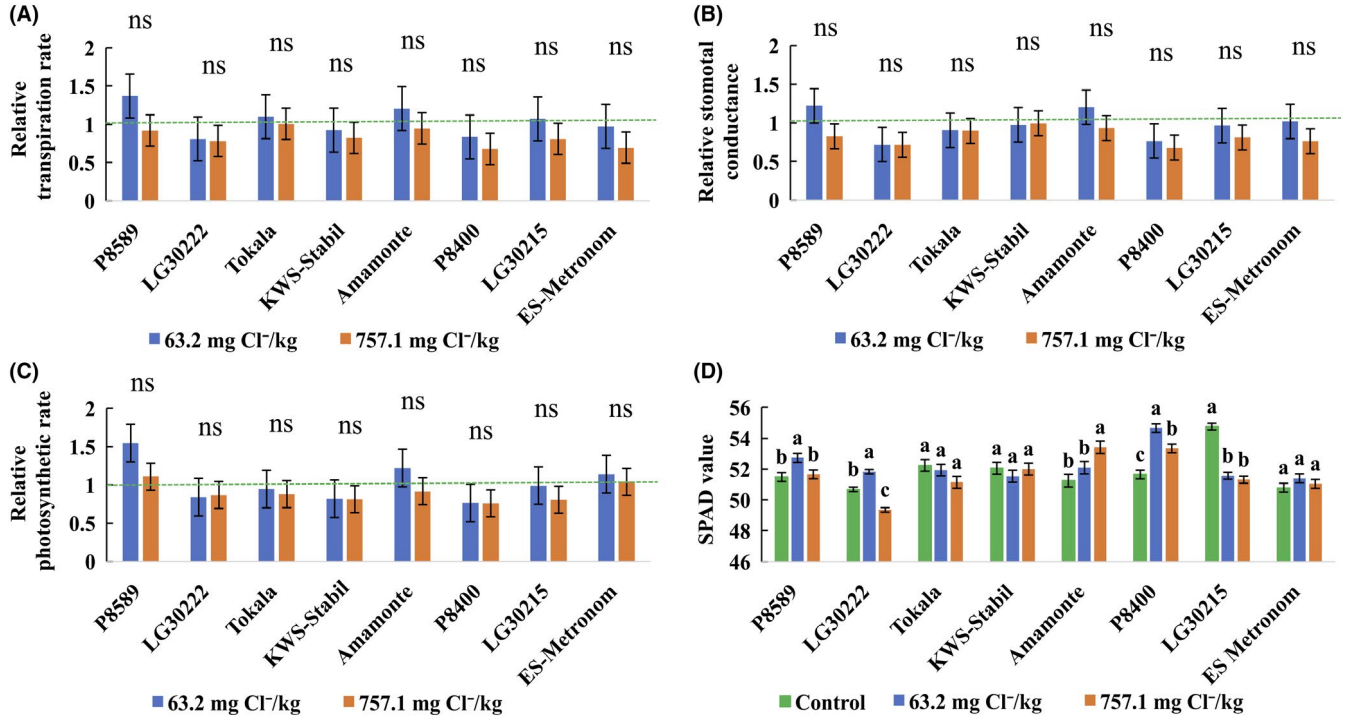

FIGURE 9 Transpiration rate, stomatal conductance, photosynthetic rate and SPAD value in the 9th leaf blade. Relative transpiration rate, relative stomatal conductance and relative photosynthetic rate were expressed as low treatment/control- and high treatment/control-ratios; * and ** indicate significant mean differences ($p < 0.05$ and $p < 0.01$, respectively) between 63.2 mg Cl^-/kg and 757.1 mg Cl^-/kg for each genotype by *T* test. Small letters indicate significant mean difference ($p < 0.05$) in SPAD readings under different treatments per genotype by Duncan Test. The sign "ns" means non-significant. (A) Relative transpiration rate of 9th leaf blade, (B) Relative stomatal conductance of 9th leaf blade, (C) Relative photosynthetic rate of 9th leaf blade, (D) SPAD value of 9th leaf blade

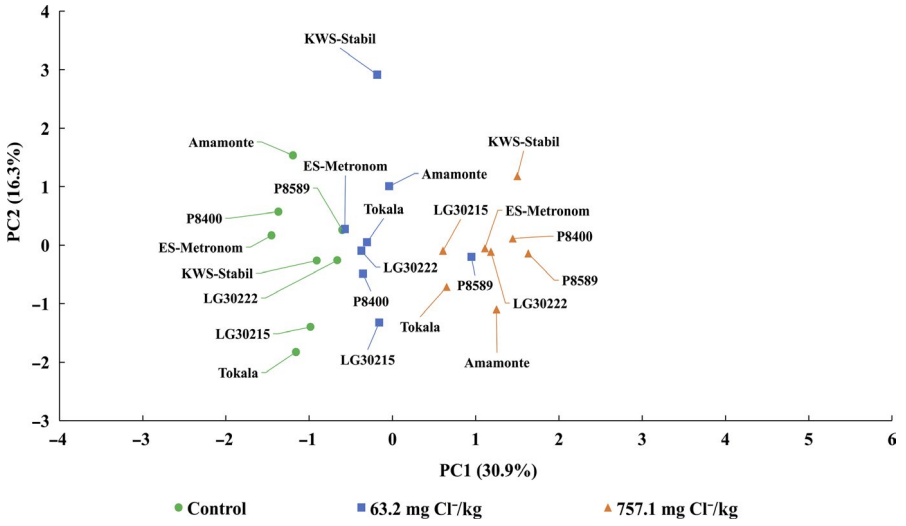

FIGURE 10 Principal component analysis (PCA) of eight maize genotypes under different treatments. In general, there are 24 parameters used for PCA analysis. They contain fresh weight in the 9th leaf blade, dry weight in the 9th leaf blade, fresh weight in other leaf blades, dry weight in other leaf blades, fresh weight in leaf sheaths, dry weight in leaf sheaths, dry weight in roots, stomatal conductance in stress induction phase (45th day to 53rd day), stomatal conductance in full stress phase (54th day to 64th day), photosynthetic rate in stress induction phase (45th day to 53rd day), photosynthetic rate in full stress phase (54th day to 64th day), transpiration rate in stress induction phase (45th day to 53rd day), transpiration rate in full stress phase (54th day to 64th day), water content in stress induction phase (45th day to 53rd day), water content (54th day to 64th day), soil electrical conductance, electrolyte leakage, osmolarity, soil pH, Cl^- concentration in the 9th leaf blade, Cl^- concentration in other leaf blades, Cl^- concentration in leaf sheaths, Cl^- concentration in roots and Cl^- concentration in the soil

Cl^- concentration reduces the photosynthetic capacity and quantum yield in *Vicia faba* due to chlorophyll degradation which may result from a structural impact of high Cl^- concentration on PSII (Tavakkoli, Rengasamy, Rengasamy, & McDonald, 2010). In our study, the reduced chlorophyll concentration did not inhibit the photosynthesis rate in maize (Figure 9C,D). This might be attributable to the fact that the

given chlorophyll concentration, as indicated by the SPAD readings, is adequate for fulfilling the photosynthetic function in our control and stressed plants, spanning from 50.7 in LG30222 to 54.7 in LG30215 among total tested genotypes. A critical SPAD value of 48.6 ± 3.8 (mean value ± standard deviation) at vegetative stage (10th leaf) was suggested to be adequate to achieve high corn yield in a field condition (Sunderman, Pontius, Pontius, & Lawless, 1997). Besides, the maximum reduction of chlorophyll, as averaged over all maize genotypes (Figure 9D), was 6.3% under high Cl^- treatment in LG30215. For comparison: under water stress, a reduction of leaf chlorophyll concentration about 40% was still not severe enough to negatively affect photosynthetic rate at mid-day in maize (Sanchez, Hall, Hall, Trapani, & Hunau, 1983).

5 | CONCLUSIONS

Chloride is not harmful when reaching concentrations as high as 757.1 mg Cl^-/kg soil DM, except for the Cl-sensitive genotype P8589 that showed leaf edge necrosis. While an equimolar sodium concentration would affect biomass, photosynthesis rate or water content, the same parameters were not affected by Cl salinity. Data show that chloride root-to-shoot translocation is restricted in most maize genotypes, indicating that maize excludes Cl^- at the xylem, which might be useful for avoiding accumulation in the photosynthetic active leaf blades. The more Cl^- sensitive genotypes accumulated more Cl^- in the shoot compared to the more tolerant ones, viz. had a smaller shoot-to-root ratio.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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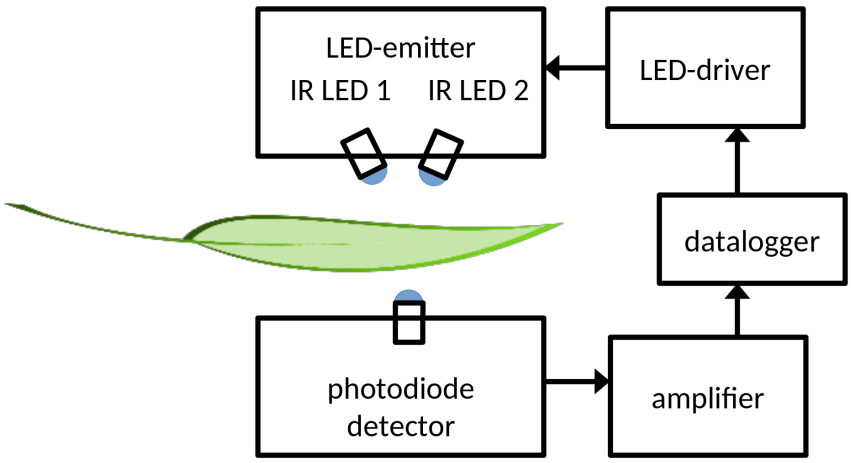
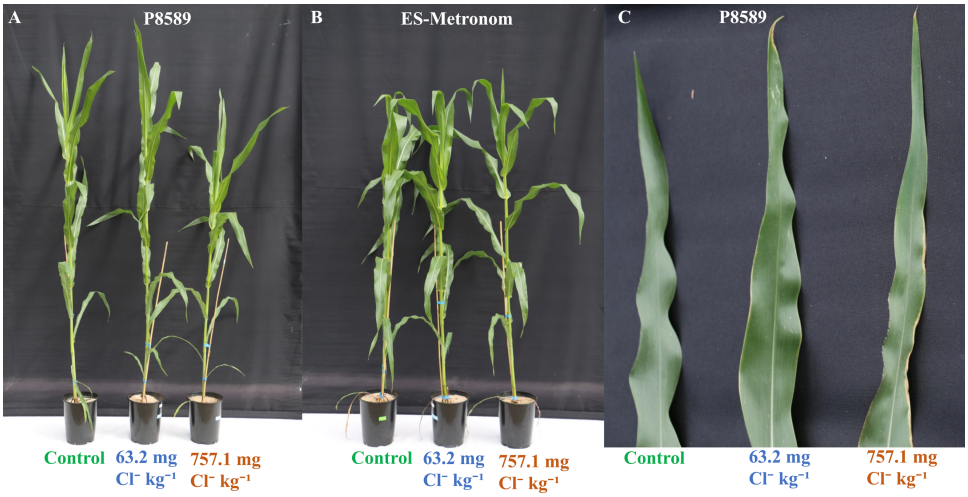
Supplementary materials



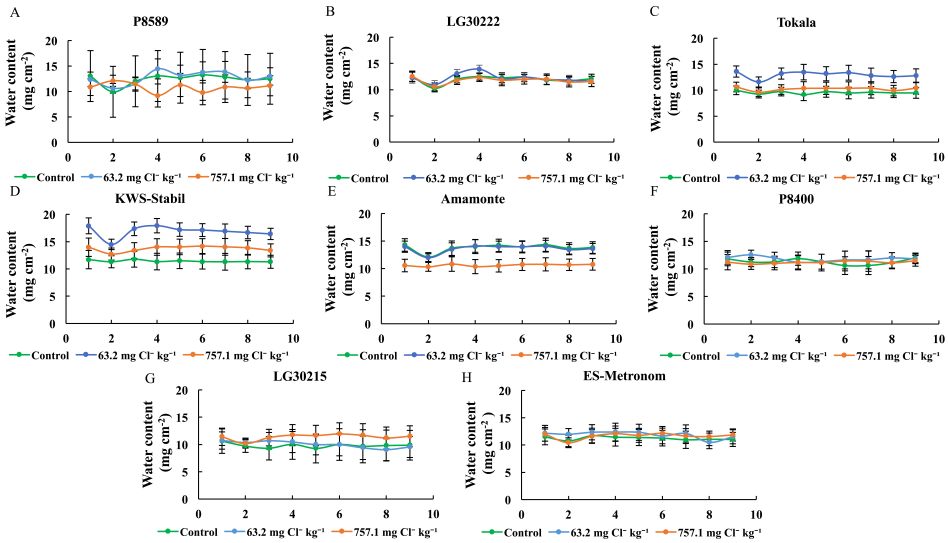
Supplementary Figure. S1 The working diagram of water sensor

A water content sensor based on infrared light emitting diodes (LED) and a photodiode linked to a custom device were calibrated against maize leaves and employed for non-invasive measurements of leaf water contents. The underlying principle is the transmission recording of two near IR-wavelengths with different absorption of water penetrating the leaf at an angle of 45°. The ratio of transmission at these two wavelengths is linearly correlated with leaf water content.



Supplementary Figure. S2 Plant phenotypes of contrasting genotypes and chlorotic symptoms in the genotype P8589

Plant phenotype of Cl^- more sensitive genotype P8589 under control, 63.2 mg Cl^- kg^{-1} soil DM and 757.1 mg Cl^- kg^{-1} soil DM (A), Plant phenotype of Cl^- more tolerant genotype ES-Metronom under control, 63.2 mg Cl^- kg^{-1} soil DM and 757.1 mg Cl^- kg^{-1} soil DM (B) and chlorotic symptoms of the 9th leaf blade in Cl^- more sensitive genotype P8589 under control, 63.2 mg Cl^- kg^{-1} soil DM and 757.1 mg Cl^- kg^{-1} soil DM (C). All plants were harvested at 10 days after treatment, that was the 64th day after sowing in loamy sandy soil.



Supplementary Figure. S3 The kinetic change of absolute water content of the 9th leaf blade

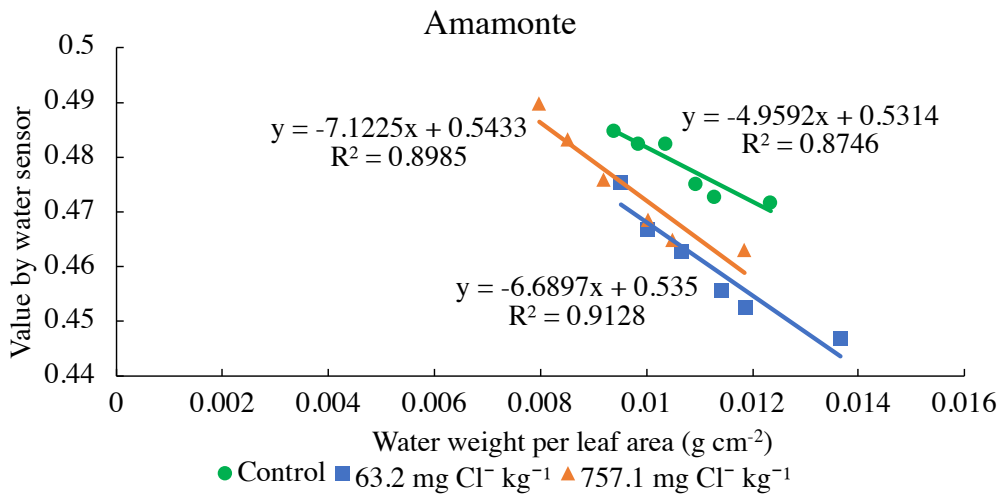
The kinetic change of absolute water content of the 9th leaf blade in 8 tested maize genotypes: P8589 (A), LG30222 (B), Tokala (C), KWS-Stabil (D), Amamonte (E), P8400 (F), LG30215 (G) and ES-Metronom (H). The water content was determined at consecutive 9 days after treatment. The value was expressed as mean ± standard error (n=5).

Supplementary Table S1 Calibration data and standard curve as exemplified by the genotype Amamonte

A

Amamonte	weight of water / S (g cm^{-2})	water value
Control-1st time point	0.012346045	0.47192
Control-2nd time point	0.011279597	0.47298
Control-3rd time point	0.010925975	0.47518
Control-4th time point	0.010349014	0.48265
Control-5th time point	0.009833471	0.48257
Control-6th time point	0.009396097	0.4849
63.2 mg Cl^- kg^{-1} -1st time point	0.013661026	0.4469
63.2 mg Cl^- kg^{-1} -2nd time point	0.011870976	0.45265
63.2 mg Cl^- kg^{-1} -3rd time point	0.011421977	0.45583
63.2 mg Cl^- kg^{-1} -4th time point	0.010651839	0.46294
63.2 mg Cl^- kg^{-1} -5th time point	0.01003781	0.46678
63.2 mg Cl^- kg^{-1} -6th time point	0.009518933	0.4756
757.1 mg Cl^- kg^{-1} -1st time point	0.011850253	0.46328
757.1 mg Cl^- kg^{-1} -2nd time point	0.010493014	0.46512
757.1 mg Cl^- kg^{-1} -3rd time point	0.010038073	0.46854
757.1 mg Cl^- kg^{-1} -4th time point	0.009194538	0.47588
757.1 mg Cl^- kg^{-1} -5th time point	0.008527292	0.48327
757.1 mg Cl^- kg^{-1} -6th time point	0.007979468	0.4898

B



The genotype Amamonte was exemplified to illustrate how absolute water content was calibrated. The calibration data of weight of water per leaf disc and the corresponding water value indicated by water sensor (A) and the established calibration curve (B). Each excised leaf disc was completely saturated overnight by floating at 4°C in ddH_2O water, then the water content was determined at six time points (0, 10, 20, 30, 45, 60 min) after the removal from the water bath during the drying process. NIR-transmission ratio and gravimetrically measured leaf water content were determined simultaneously, yielding a linear calibration curve of leaf water content versus NIR-ratio specific for maize leaves.

Supplementary Table S2 PCA parameters and scoring analysis

VAR00001	Parameters
VAR00002	Fresh weight in the 9th leaf blade
VAR00003	Dry weight in the 9th leaf blade
VAR00004	Fresh weight in other leaf blades
VAR00005	Dry weight in other leaf blades
VAR00006	Fresh weight in leaf sheaths
VAR00007	Dry weight in leaf sheaths
VAR00008	Dry weight in the root
VAR00009	1st* phase stomatal conductance
VAR00010	2nd* phase stomatal conductance
VAR00011	1st* phase photosynthetic rate
VAR00012	2nd* phase photosynthetic rate
VAR00013	1st* phase transpiration rate
VAR00014	2nd* phase transpiration rate
VAR00015	1st* phase water content
VAR00016	2nd* phase water content
VAR00017	Soil electrical conductance
VAR00018	Electrolyte leakage
VAR00019	Osmolarity
VAR00020	Soil pH
VAR00021	Cl^- concentration in the 9th leaf blade
VAR00022	Cl^- concentration in other leaf blades
VAR00023	Cl^- concentration leaf sheaths
VAR00024	Cl^- concentration in the root
VAR00025	Cl^- concentration in the soil

* 1st phase means stress induction phase (45th day to 53rd day); 2nd phase means full stress phase (54th day to 64th day); The data of each parameter was the mean value of 5 biological replicates.

Component Matrix[a]

	Component						
	1	2	3	4	5	6	7
VAR00024	-.888						
VAR00023	-.884						
VAR00021	-.860						
VAR00025	-.855						
VAR00017	-.828						
VAR00022	-.740						
VAR00019	-.654						
VAR00014							
VAR00005		.732					
VAR00006		.678					
VAR00004		.676					
VAR00010		-.671					
VAR00008		.655					
VAR00007							
VAR00016			.856				
VAR00015			.853				
VAR00003			-.848				
VAR00002			-.829				
VAR00012				.697			
VAR00009				-.615			
VAR00020							
VAR00011					.698		
VAR00013					.626		
VAR00018							-.623

Extraction Method: Principal Component Analysis.

a. 7 components extracted.

Rotated Component Matrix[a]

	Component						
	1	2	3	4	5	6	7
VAR00022	.943						
VAR00021	.923						
VAR00023	.919						
VAR00024	.863						
VAR00025	.857						
VAR00017	.705						
VAR00015		.918					
VAR00016		.908					
VAR00003		-.840					
VAR00002		-.802					
VAR00004			.893				
VAR00005			.879				
VAR00019			-.719				
VAR00020				.817			
VAR00007				.777			
VAR00006				.746			
VAR00008				.704			
VAR00012					.917		
VAR00014					.835		
VAR00010					.730		
VAR00011						.934	
VAR00013						.902	
VAR00009						.662	
VAR00018							.734

Extraction Method: Principal Component Analysis.

Rotation Method: Varimax with Kaiser Normalization.

a. Rotation converged in 10 iterations.

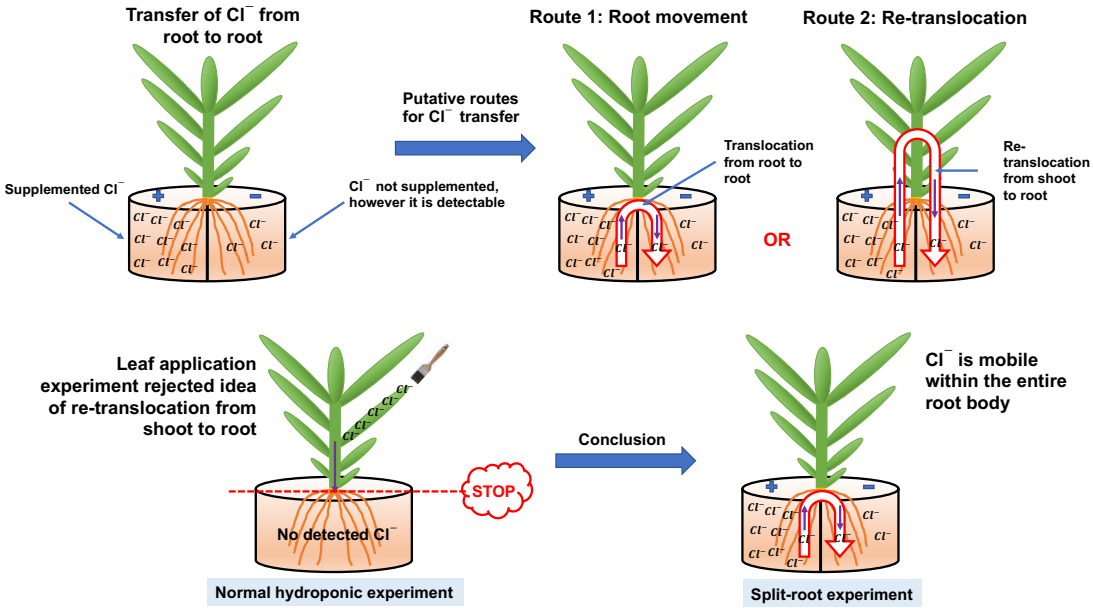
Supplementary Table S3 Soil pH of eight maize genotypes

Genotypes	Control	63.2 mg Cl^- kg^{-1}	757.1 mg Cl^- kg^{-1}
P8589	7.29±0.01a	7.28±0.01a	7.30±0.01a
LG30222	7.20±0.01a	7.21±0.01a	7.21±0.01a
Tokala	7.09±0.03a	7.10±0.03a	7.14±0.03a
KWS-Stabil	7.25±0.01a	7.26±0.01a	7.23±0.01a
Amamonte	7.29±0.02a	7.24±0.02a	7.26±0.02a
P8400	7.27±0.01a	7.27±0.01a	7.29±0.01a
LG30215	7.28±0.02a	7.29±0.02a	7.26±0.02a
ES-Metronom	7.27±0.01a	7.25±0.02a	7.27±0.01a

The data of soil pH were expressed as mean value ± standard error. Small letters indicate significant mean difference ($P<0.05$) under different treatments per genotype.

5. Chapter II

The root as a sink for chloride under chloride-salinity



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Research article

The root as a sink for chloride under chloride-salinity

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ABSTRACT

Maize has to avoid excess tissue accumulation of Cl^- to withstand conditions of Cl^--salinity. Restriction of loading of Cl^- into the root xylem is one mechanism to keep shoot Cl^--concentrations low. The proportion of Cl^- that reaches the shoot has to be stored away from the primary site of photosynthesis and growth. We tested whether or not maize is able to re-translocate significant amounts of Cl^- from shoot back to root and out into the rooting media. Ion analysis revealed that maize cannot re-translocate Cl^-; however, it is stored in sheaths of the old leaves and, surprisingly, in roots. Sequestration of Cl^- in the roots might be a strategy to keep concentrations low in young growing shoot tissues and in leaf blades where photosynthesis is running.

1. Introduction

In maize (*Zea mays* L.), excess supply of chloride (Cl^-) may induce toxicities in the cytosol (Geilfus, 2018a) if not sequestered into the leaf vacuoles (Lohaus et al., 2000). Such a vacuolar compartmentation of Cl^- was also shown for grapevine (*Vitis vinifera* L. cv. Biancone) (Storey et al., 2003), barley (*Hordeum vulgare* L. cv. Klondike) (Britto et al., 2004) and other plants (Li et al., 2017; Geilfus, 2018a). However, if the vacuolar storage capacity is exceeded, Cl^- may accumulate in the cytosol, which is thought to affect enzyme activities and cellular water balance (Rausch et al., 1996; Geilfus, 2018b). In order to avoid accumulation of excess of Cl^- in the cytosol, the anion can be sequestered in tissue that is not the site of primary photosynthesis and growth (Tavakkoli et al., 2010; Teakle and Tyerman, 2010). For example, plants are able to store Cl^- in leaf base, stem/sheath and old tissues such as barley and sorghum (Boursier et al., 1987; Munns, 1985).

Studies on sodium-salinity have revealed that plants acquired many further strategies to cope with high level of salts. The following mechanisms are known to avoid excessive cellular accumulation of sodium: the restriction of loading of sodium into the root xylem to avoid acropetal transport to the shoot (Tester and Davenport, 2003), shoot re-translocation of sodium back to the root (Lessani and Marschner, 1978), sodium exclusion out of the root in the rooting solution (Munns, 2005), storage of sodium in stem and partitioning to petiole rather than mesophyll (Munns, 2005). The re-translocation of sodium from the plant tissue back into the rooting solution is believed being a powerful mechanism to maintain tissue sodium concentrations low (Lessani and Marschner, 1978; Munns and Fisher, 1986; Wolf et al., 1990; Durand and Lacan, 1994; Gouia et al., 1994; Jeschke et al., 1995). There is evidence that plants like mung bean (*Vigna radiata* L. cv. Berken) (Salim, 1988), tomato (*Solanum lycopersicum* L.) (Olías et al., 2009), rice (*Oryza sativa* L.) (Yeo and Flowers, 1982) or *Arabidopsis thaliana* (Berthomieu et al., 2003) are able to re-translocate excess of Na^+ from the plant tissue back in the rooting solution. This is discussed being a mechanism that helps the plant to endure sodium-salinity by keeping Na^+ concentrations low; nevertheless, effectiveness in the field is still debated (Lessani and Marschner, 1978; Yeo and Flowers, 1982).

However, the situation with regard to the ability of plants to re-translocate Cl^- from the tissue back into the rooting solution under conditions of Cl^- salinity, or at least to store Cl^- within the root is unclear. It is not known whether Cl^- can be re-translocated and, secondly, whether or not this would be effective to keep shoot tissue concentrations low. For barley (*Hordeum vulgare* L.), Munns and Fisher (1986) have shown that this mechanism might exist, however, not being effective as the amount of basipetally translocated Cl^- is by far too low. Labelled Cl^- has been used in a study on barley but it was concluded that release of shoot-sourced Cl^- into the external rooting solution was not sufficient to keep tissue Cl^- concentration low (Greenway and Thomas, 1965). Nevertheless, it tended to be found that a re-translocation of Cl^- seemed to function a little bit better in maize plants compared to other species such as bean (*Phaseolus vulgaris* L.), cress (*Lepidium sativum* L.), pepper (*Capsicum annuum* L.), sugar beet (*Beta vulgaris* L.), safflower

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(*Carthamus tinctorius* L.) and sunflower (*Helianthus annuus L.*) (Lessani and Marschner, 1978). A tracer study with ^{36}Cl witnessed for maize that the anion could be re-translocated to the apical part (more than 40%), to the basal part of the leaf plus stem (more than 40%), to the shoot apex (around 6%) and to the root (around 6%). However, only a tiny amount (less than 3.5%) was secreted to the external medium, which might be too less to relieve the plant. Both tested maize genotypes were considered as tolerant to a 100 mM NaCl stress (Lessani and Marschner, 1978). Similarly, higher phloem transport rates of Cl^- were observed in maize plants than barley or spinach (*Spinacia oleracea* L.), probably attributable due to its phloem-based higher carbon translocation rate as C4 plants (Lohaus et al., 2000).

To clarify the central questions whether under conditions of Cl^--salinity maize is able to (i) sequester Cl^- in the root or to (ii) secrete it back into the rooting solution, the presented study was carried out. We used calcium and/or magnesium as Cl^- accompanying counter cations. Thus, sodium effects are excluded in this study.

2. Materials and methods

2.1. Plant materials, cultivation and experimental design

In our previous study, the two maize genotypes P8589 (Pioneer Hi-Bred Northern Europe Sales Division GmbH) and ES-Metronom (Euralis Saaten GmbH) were demonstrated to contrast in their ability to restrict the uptake of Cl^- to the shoot under conditions of Cl^- salinity. P8589 is a less efficient Cl^- excluder while ES-Metronom excludes more efficiently (Zhang et al., 2019). This pair of contrasting maize plants was used to investigate the relation between uptake of Cl^-, sequestration and translocation. Using these genotypes, three different experiments were carried out. For the first one, plants were cultivated in soil that was supplemented with excess of Cl^-. Aim was to understand whether and if so where excess of Cl^- is stored. The second experiment was a hydroponic split-root experiment. It was tested whether Cl^- is excluded into the rooting medium, if maize takes up high amounts of Cl^-. In a follow-up experiment, maize was cultivated hydroponically in a normal pot (not split-root device) without excess of Cl^-. Leaves were brushed with a high dose of Cl^- to investigate whether this foliar applied Cl^- accumulates in the root or the rooting medium.

2.1.1. Soil cultivation with excess of Cl^-

The two contrasting genotypes P8589 (less efficient Cl^- excluder) and ES-Metronom (more efficient Cl^- excluder) grew for nine weeks in a greenhouse of University of Hohenheim. During the entire growth period, the average humidity and temperature were 64.1% rF and 23.1 °C at the whole day, recorded by Mini-Datalogger testo 174H (Testo SE & Co. KGaA, Neustadt, Germany). The plants were cultivated in 7 L Mitscherlich pots (one plant per pot) being filled with 7840 g (DM) soil mixture. The mixed soil was composed of 47.5% (w/w) subfloor loam soil (C_{org}, 4.0%; Ostfildern, Stuttgart), 47.5% (w/w) sand of particle size 0–2 mm and 5% (w/w) sour turf soil (Baywa, Filderstadt). The initial pH of this soil mixture was 7.06. Extra sand (600 g) was used to cover the soil surface to minimize evaporation. The sandy soil contained 8.8 $mgCl^-\ kg^{-1}$ soil DM. The watering amount was adjusted to maintain 70% (w/w) water holding capacity (WHC) of the potted soil. Pots were fertilized with 2 g NH_4NO_3, 5 g KH_2PO_4, 2 g $MgSO_4*7H_2O$ and 0.3 g Fetrilon-combi micronutrient solution (AgNova Technologies Pty Ltd). This solution contained micronutrients (1.5% boron, 0.6% copper, 4.0% iron, 3.0% manganese, 0.05% molybdenum and 4.0% zinc) and some macronutrients (0.8% magnesium and 1.3% sulphur). Micronutrient requirements for Cl^- were fulfilled by the natural abundance of Cl^- in the subfloor loam soil. At 45 days after emergence, 252.4 mg Cl^- were given per kg dry soil. Cl^- was given as $CaCl_2*2H_2O$. This amount was gradually increased to a Cl^- dose of 757.1 mg Cl^- per kg dry soil by adding 126.2 mg Cl^- per kg dry soil every second day. Finally, pots contained 6.4 g of Cl^- (controls without Cl^- contained 8.8 mg of Cl^- per kg soil). This concentration was found suitable for inducing Cl^--stress (Zhang et al., 2019) and is unlikely to induce Ca toxicity as a leaf Ca concentration of 0.8% (w/w, DM) is in the optimal range that spans from 0.5% to 1.6% (Gaj et al., 2018). Plants grew 10 days after full stress treatment was applied. For analyzing how Cl^- was distributed in the plant, plant was divided into 11 fractions: young leaf blades, young leaf sheaths, leaf tip, leaf edges, leaf center, leaf base, leaf midrib, leaf sheath, old leaf blades, old leaf sheaths and root (see Fig. 1A). Cl^- treated plants were grown in four biological replicates (n = 4).

2.1.2. Split-root experiment

Maize seeds from the two contrasting genotypes P8589 (less efficient Cl^- excluder) and ES-Metronom (more efficient Cl^- excluder) were soaked in 1 mM $CaSO_4$ solution for 24 h and then germinated in the filter paper for 7 days. At the 2-leaf stage, maize seedlings were transferred to a hydroponic split root device using 4.5 L of our half strength nutrient solution (nutrient concentration below). The split-root device had two chambers for the nutrient solution. The roots were distributed evenly across both chambers. After 7 days of cultivation, ion strength was increased to full strength nutrient solution. The water used for hydroponic solution was deionized. MES buffer (1 mM) was used to clamp the hydroponic pH in the range of 6.2–6.5 by using KOH. The experiment was run in the greenhouse of University of Hohenheim. During the entire growth period, the average humidity and temperature were 58.7% rF and 24.5 °C at the whole day.

The nutrient solution had the following concentration, being modified from the recipe by (Zörb et al., 2015). Macronutrients (mM): KH_2PO_4, 2.4; K_2SO_4, 1; $MgSO_4*7\ H_2O$, 2; and Fe-EDTA, 0.2. Micronutrients (μM): H_3BO_3, 5; $MnSO_4*H_2O$, 2; $ZnSO_4*7H_2O$, 0.5; $CuSO_4$, 0.01; $CaCl_2$, 0.3; $(NH_4)_6Mo_7O_{24}*4H_2O$, 0.005. The split root experiment was conducted under two nitrate (NO_3^-) concentrations; the reason for this is explained in the discussion-section (in brief: we wanted to induce an uptake competition between Cl^- andNO_3^-). For this, either 1 mM Ca $(NO_3)_2*4H_2O$ and 0.3 mM $(NH_4)_2HPO_4$ (low NO_3^- treatment) or 4 mM $Ca(NO_3)_2*4H_2O$ and 1.3 mM $(NH_4)_2HPO_4$ (high NO_3^- treatment) were given. All variants have received sufficient phosphorus via adding 2.4 mM KH_2PO_4. This was to make sure that there is no effect of phosphorus deficiency. The nutrient solutions were renewed once a week.

Cl^- stress was initiated at the 21st day after transfer of 2-leaf-stage seedlings into nutrient solution by adding 40 mM of Cl^- via 10 mM $CaCl_2*2H_2O$ and 10 mM $MgCl_2*6H_2O$ into one chamber (the other remained free of excess Cl^-). Cl^- was given together with both calcium and magnesium to avoid any toxicities from the one or the other counter cation. Concentration was increased stepwise about 40 mM on the two consecutive days to a final concentration of 120 mM Cl^- at the 23rd day. The pH of the nutrient solution was daily adjusted to 6.5 in both chambers. Once a Cl^- concentration of 120 mM was reached in the one part of the chamber, plants grew for 3 days before experiment was stopped at the 26th day. Plants from this experimental group are named "split-root" plants. Root samples from the chamber that was supplemented with excess of Cl^- were labelled with a plus symbol that indicates exposure to excess Cl^-, while a minus symbol indicates that roots were not treated with excess of Cl^- (i.e. "(±)" means that the left chamber of the split-root device was with and the right one without excess of Cl^-). The experiment was composed of two further control groups: in the positive control, both chambers were supplemented with 120 mM of Cl^- in the same way as described above (a split root device was used but nutrient solution was the same in both chambers). Root samples from these plants are labelled as "positive control" being abbreviated as "PC". In the negative control, both chambers were not supplemented with excess of Cl^- (a split root device was used but nutrient solution was the same in both chambers). Root samples from these plants are labelled as "negative control" being abbreviated as "NC". The addition of Ca or Mg as Cl^--accompanying cation did not cause an excessive accumulation of both cations because in the positive

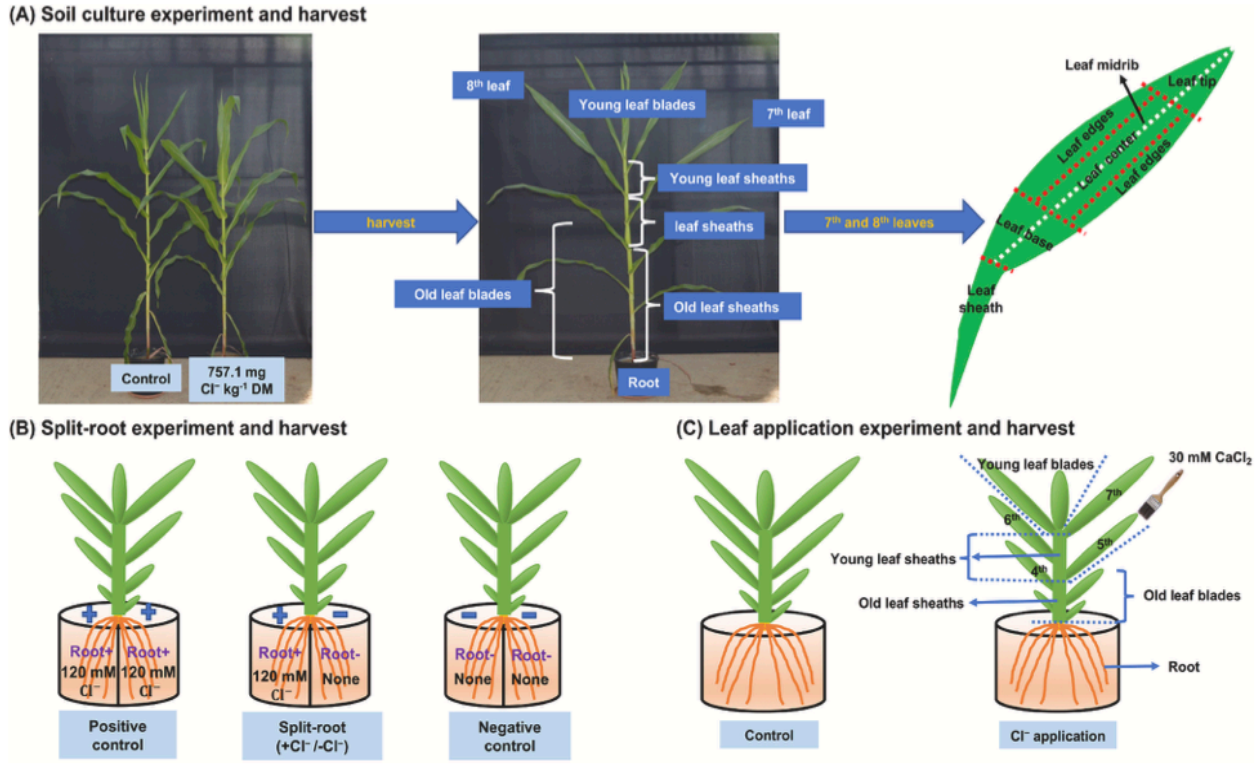

Fig. 1. Overview of experimental design. Soil culture experiment (A), split-root experiment (B), leaf application experiment (C). Illustrations shows fractionation of plant material for further analysis.

controls, Ca and Mg concentrations were 0.5% and 0.4%, respectively. This is in the optimal range of 0.5%–1.6% for Ca and 0.3%–0.6% for Mg (Gaj et al., 2018). All groups were grown in quadruplicate (n = 4 biological replicates) in the greenhouse (see Fig. 1B for details).

2.1.3. Leaf application experiment

Maize seeds of the contrasting genotypes were germinated in filter paper as done for the split-root experiment. Seedlings at the two-leaf stage were transferred to the half-strength nutrient solution which was replenished to full strength after 7 days. Deionized water was used for producing the nutrient solution. The nutrient solution was composed of macronutrients (mM) with $Ca(NO_3)_2$*$4H_2O$, 2; KH_2PO_4, 2.4; K_2SO_4, 1; $MgSO_4$*$7H_2O$, 2; $(NH_4)_2HPO_4$, 0.67 and Fe-EDTA, 0.2 and micronutrients (μM) with H_3BO_3, 5; $MnSO_4$*H_2O, 2; $ZnSO_4$*$7H_2O$, 0.5; $CuSO_4$, 0.3, $CaCl_2$, 0,01 and $(NH_4)_6Mo_7O_{24}$*$4H_2O$, 0.005. MES buffer (1 mM) was used to clamp the pH in the range of 6.2–6.5 by using KOH. The nutrient solutions were renewed once a week. At the 18th day after transferring to the hydroponic solution, 60 mM Cl^- were foliar applied onto the surface of the 5th leaf blade by brushing 0.79 ml of a 30 mM $CaCl_2$*$2H_2O$ solution twice at the same day on the adaxial and the abaxial face. The detergent of 0.15% (v/v) (agroplanta GmbH & Co., Langenpreising-Zustorf, Germany) was added to reduce surface tension of the water. This leaf application was repeated on three consecutive days until leaf edge necrosis was observed (Fig. S1). In total, 5.05 mg of Cl^- were brushed per leaf blade. This necrosis was believed to be caused by excess Cl^- rather than Ca toxicity, since Ca concentration of the leaf blade was only 1.4% (w/w) DM; optimal concentration ranges from 0.5% to 1.6% (Gaj et al., 2018). The plants grew another two days before being harvested at the 22nd day after transferring. For subsequent analysis, plant was divided into 10 fractions: young leaf blades (8th and 9th), 7th leaf blade, 6th leaf blade, 5th leaf blade, 4th leaf blade, and old leaf blades (1st, 2nd and 3rd), young leaf sheaths, old leaf blades, old leaf sheaths and root (see Fig. 1C for details). All plants were grown within four biological replicates in the greenhouse of University of Hohenheim. During the entire growth period, the average humidity and temperature were 67.7% rF and 21.9 °C at the whole day.

2.2. Methods

2.2.1. Cl^- concentration

Cl^- concentration was determined according to the chloride-meter method as described by (Zhang et al., 2019). For this, plant tissue (200 mg DM) was grinded (particle diameter of 0.5 mm) and dissolved in 10 ml ddH_2O before it was heated in a water bath at 80 °C for 15 min. After cooling down on ice for 7 min, the mixture was filtered through a cyclic filter pater (diameter of 90 mm) and flow-through was collected in a 15-ml falcon tube. 600 μl of the filtrate were mixed with 1 ml gelatin reaction (Biorapid GmbH, Umkirch, Germany) and 15 ml acid buffer (0.64% v/v nitric acid and 5.76% v/v acetic acid). A chloride-meter purchased from Eppendorf 6610 (Biorapid GmbH, Umkirch, Germany) was used to quantify Cl^- (detection range: 10–999 mg Cl^-l^{-1}). Each measurement had three technical replicates.

2.2.2. Calculation of the Cl^- translocation factor

Translocation factor (TF) was used to estimate the ability of plants to transport Cl^- from root to shoot. TF was calculated as the ratio of Cl^- concentration in shoot to Cl^- concentration in root (Eller and Brix, 2016). A ratio below 1 indicates that plant is an Cl^- excluder; the lower the ratio, the more pronounced the ability to exclude transfer of Cl^- from root to shoot.

2.3. Statistical analysis

Data analysis and plotting was done using R studio software (version 1.1.456; R studio Inc, Boston, USA). Multiple comparison was evaluated by ANOVA and LSD test with library (agricolae) (de Mendiburu and de Mendiburu, 2019) and double comparison was tested by T-test. Whisker-box graphs were plotted using ggplot2 version 2.3.3.0 (Wickham, 2016).

3. Results

3.1. Accumulation pattern of Cl^- in the shoot under conditions of Cl^--salinity

The first experiment (soil culture) revealed a Cl^- translocation factor (TF) lower than 1 for both genotypes. This indicates that both cultivars restrict acropetal transfer of excess of Cl^-. P8589 had a higher TF than ES-Metronom. Thus, the genotype P8589 was less efficient with regard to restrict transfer to Cl^- from root to shoot (Fig. 2A). This finding is consistent with our previous work (Zhang et al., 2019).

Both genotypes accumulated the most Cl^- ($P < 0.05$) in old leaf sheaths and root (Fig. 2B). The less efficient Cl^- excluder P8589 had higher Cl^- concentration ($P < 0.01$) in old leaf blades than ES-Metronom. Old leaf blades of less efficient Cl^- excluder P8589 showed higher Cl^- concentration ($P < 0.05$) than young leaf blades of the same genotype. No such significant difference was observed in the more efficient Cl^- excluder ES-Metronom (Fig. 2B).

A detailed analysis of the 7th and 8th leaf, i.e. young expanding leaf materials, revealed for both genotypes that leaf sheath had the highest Cl^- concentration ($P < 0.05$) (Fig. 3A). The descending order of rank as detected in less efficient Cl^- excluder P8589 was leaf sheath > leaf midrib > leaf base > leaf center > leaf edges > leaf tip (Fig. 3B). A similar order of rank was detected in the ES-Metronom with the exception that there was no difference between leaf center and leaf edges. Furthermore, the comparison between both genotypes revealed that the more efficientCl^- excluder ES-Metronom had lower Cl^- concentration in leaf tip ($P < 0.05$), leaf center ($P < 0.001$), leaf edges ($P < 0.05$) and leaf base ($P < 0.01$) compared to the less efficient Cl^- excluder P8589 (Fig. 3A and B).

3.2. The root as sink for Cl^-

As expected, the split-root experiment revealed the highest Cl^- concentration to be in the roots that were exposed to the highest Cl^--dose (120 mM Cl^-), i.e. in the roots from the positive control ("PC") and in the roots that grew in the split root chamber that was supplemented with excess of Cl^- ("+Cl^-") (Fig. 4A). This was true for both genotypes ($P < 0.05$). An analysis of roots from the same plant that, however, grew in the other chamber of the split-root device that was not supplemented with excess of Cl^- ("-Cl^-"), revealed a lower Cl^- concentration than in the root that grew in the other chamber that was supplemented with excess of ("+Cl^-"). This was true for both genotypes ($P < 0.05$). Surprisingly, Cl^- concentration in roots that grew in the split-root chamber "-Cl^-" was still higher ($P < 0.05$) than in the negative control ("NC") (Fig. 4A). In other words, there must have been transfer of Cl^- from the nutrient solution from the one chamber with 120 mM Cl^- ("+Cl^-") to the roots that grew in the other chamber that was not supplemented with excess of Cl^- ("-Cl^-"). This was not observed for the more efficient Cl^- excluder ES-Metronom: here Cl^- concentration in the roots that grew in the side of the split-root chamber that was not supplemented with excess of Cl^- ("-Cl^-") was as low as in the roots that in the negative control ("NC") ($P < 0.05$; Fig. 4A).

Next, we measured Cl^- concentration in the nutrient solutions. This was done to see if any of the genotypes was able to secrete a significant amount of Cl^- into the nutrient solution of the split-root chamber that was not supplemented with a high load of Cl^-. However, a comparison between both chambers, i.e. the "+Cl^-" and "-Cl^-" site of the split root device, revealed that this is not the case: Cl^- was not detectable in the nutrient solution of the "-Cl^-"-chamber (Fig. 4B). Nevertheless, for less efficient Cl^- excluder P8589, this analysis revealed that the Cl^- concentration in the nutrient solution of the positive control ("PC") was higher compared to the Cl^- concentration in the nutrient solution of the split root chamber that was supplemented with the same concentration of Cl^-, i.e. 120 mM ("+Cl^-") ($P < 0.01$). This was not observed for the more efficient Cl^- excluder ES-Metronom. Here, the Cl^- concentration in the nutrient solutions of the positive control ("PC") and the "+Cl^-" of split-root chambers were identical.

In a follow-up experiment, we repeated this experiment with a nutrient solution that was identical with regard to Cl^- concentration but, importantly, contained a higher nitrate (NO_3^-) concentration. The data presented in Fig. 4 are based on a nutrient solution composed of 2 mM NO_3^-, data from the follow-up next experiment presented in Fig. 5 are based on a nutrient solution composed of 8 mM NO_3^-. This NO_3^- concentration is certainly high and unrealistic to occur in nature; however, it presents a nice experimental tool to induce a NO_3^--based uptake competition between NO_3^- and Cl^-. Such a competition is known to exist because under conditions of high concentration of Cl^-, Cl^- and NO_3^-

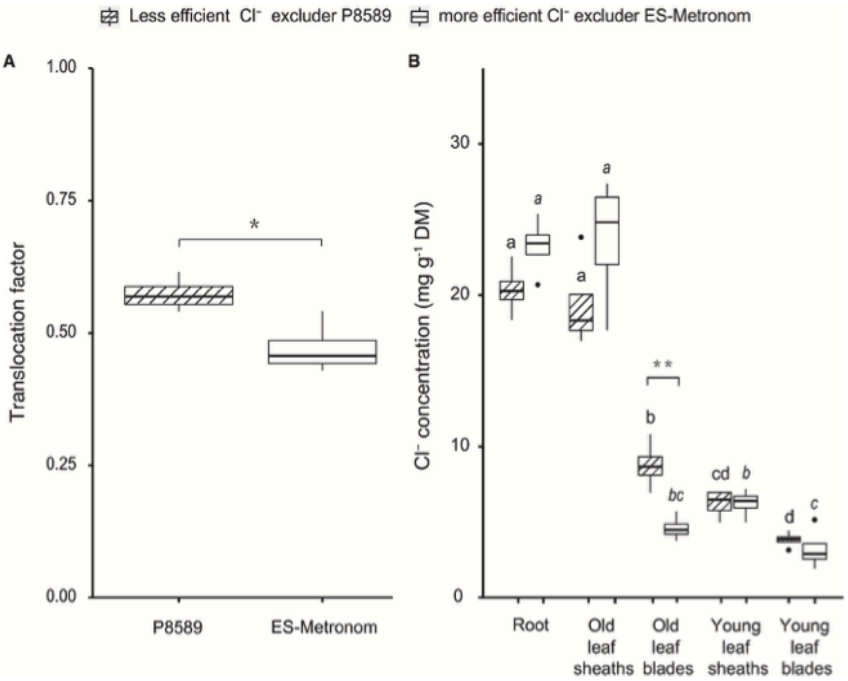

Fig. 2. Translocation factor and tissue Cl^- concentration. Translocation factor of Cl^- (A), tissue concentration of Cl^- (B). P8589, less efficient Cl^- excluder (slashed box-whisker-plot); ES-Metronom, more efficient Cl^- excluder (clean box-whisker-plot). Asterisks in (A) and (B) represent significant differences (*, $P < 0.05$; **, $P < 0.01$) by T-test between P8589 and ES-Metronom. Small letters (regular font for P8589 and italic font for ES-Metronom) in (B) show significant differences by LSD-test between plant tissues of the same genotype. Data are based on 4 biological replicates.

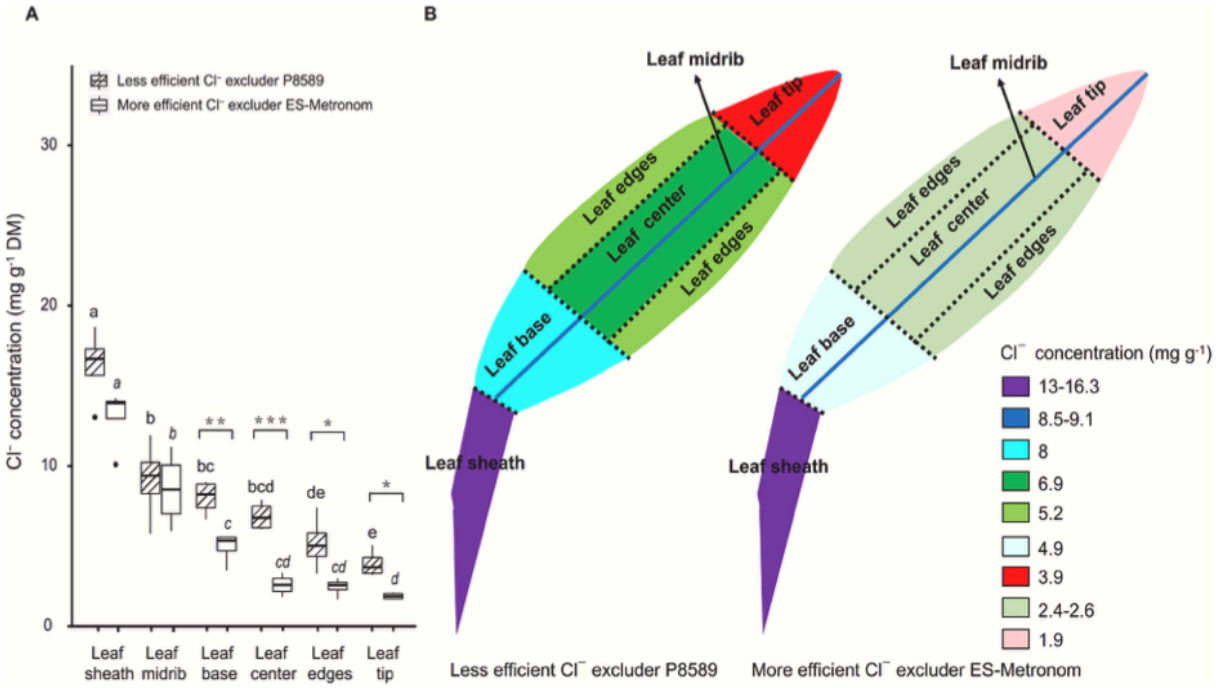

Fig. 3. Cl^- distribution in young leaves. Cl^- concentration in shoot (A), color-coded visualization of Cl^- concentration pattern (B). P8589, less efficient Cl^- excluder (slashed box-whisker-plot); ES-Metronom, more efficient Cl^- excluder (clean box-whisker-plot). Asterisks in (A) represent significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) by T-test between P8589 and ES-Metronom. Small letters (regular font for P8589 and italic font for ES-Metronom) in (A) show significant differences by LSD-test between plant tissues of the same genotype. Data are based on 4 biological replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

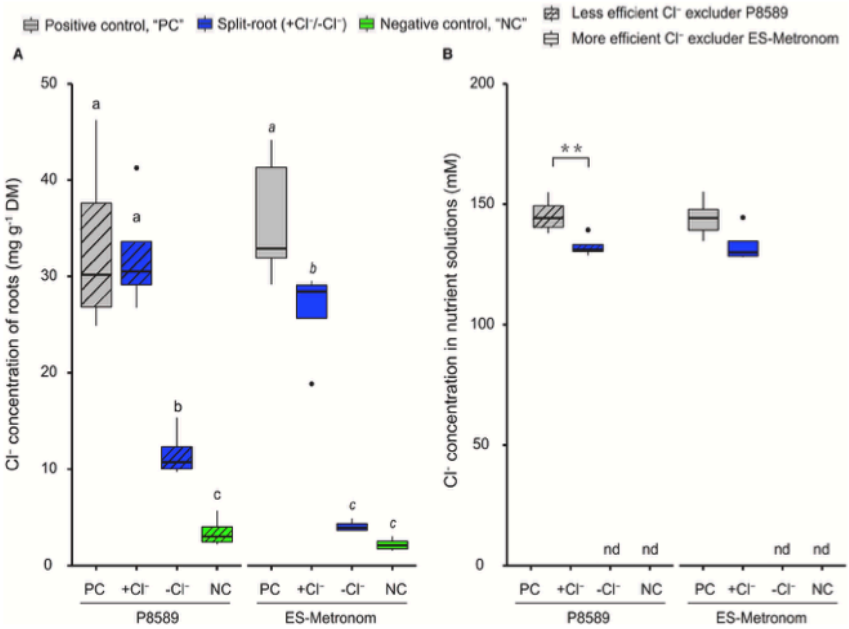

Fig. 4. Cl^- concentration in roots and nutrient solutions under low NO_3^- condition (2 mM). Cl^- concentration in roots (A), Cl^- concentration in nutrient solutions (B). P8589, less efficient Cl^- excluder (slashed box-whisker-plot); ES-Metronom, more efficient Cl^- excluder (clean box-whisker-plot). "Positive control" (abbreviated as "PC"), 120 mM Cl^- was supplemented into the nutrient solution. "+Cl^-", this abbreviation labels the chamber of the split-root device that was supplemented with 120 mM Cl^-. "-Cl^-", this abbreviation labels the chamber of the split-root device that was not supplemented with excess of Cl^-. "Negative control" (abbreviated as "NC"), no excess of Cl^- was supplemented into the nutrient solution. Small letters (regular font for P8589 and italic font for ES-Metronom) in (A) indicate significant differences by LSD test ($P < 0.05$) between different treatments in same genotypes. Asterisks in (B) represent significant differences (*, $P < 0.05$; **, $P < 0.01$) by T-test between P8589 and ES-Metronom. "nd", not detectable. Data are based on 4–8 biological replicates.

uptake is both facilitated by the activity of a NO_3^- transporter that belongs to the PTR family: *Zm*NPF6.4 is a transmembrane protein that is located in the plasma membrane of root cells. When external Cl^- concentration was increased from 0 to 10 mM, Wen et al. (2017) have demonstrated that *Zm*NPF6.4 also facilitates uptake of Cl^-, which explains the competition to NO_3^- uptake. In our results, major effects of treatment and genotype were the same as observed under conditions of 2 mM NO_3^- (Figs. 4A and 5A). Key differences when subjected to different NO_3^--concentrations were the following: First, Cl^- concentrations of the roots were lower under condition of 8 mM NO_3^- (compare Fig. 4A versus Fig. 5A). Second, for the less efficient Cl^- excluder P8589, the difference in the Cl^- concentration of the nutrient solution between positive control ("PC") and the split root chamber that was also supplemented with 120 mM Cl^-, i.e. 120 mM ("+Cl^-"), was not that pronounced as under condition of 2 mM NO_3^- (compare Fig. 4B versus Fig. 5B).

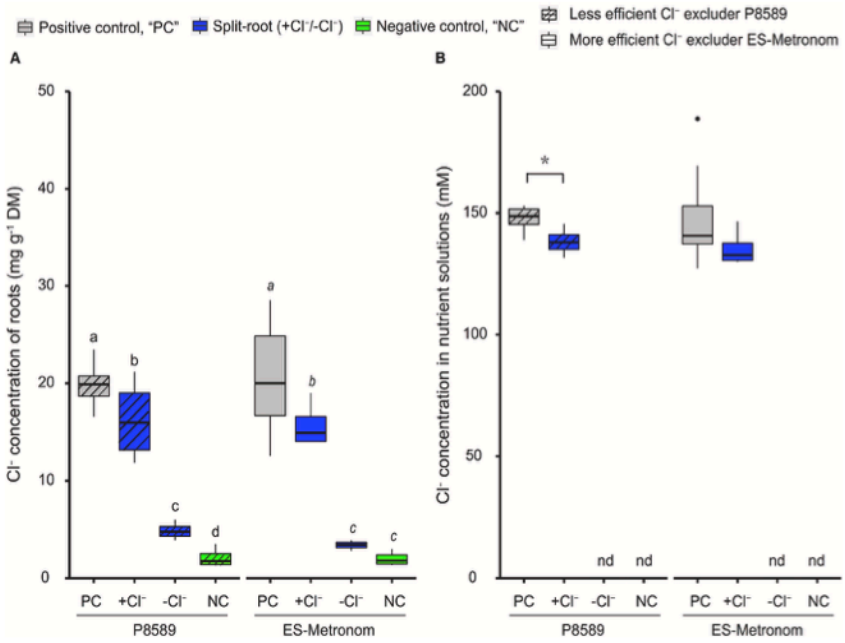

Fig. 5. Cl^- concentration in roots and nutrient solutions under high NO_3^- condition (8 mM). Cl^- concentration in roots (A), Cl^- concentration in nutrient solutions (B). P8589, less efficient Cl^- excluder (slashed box-whisker-plot); ES-Metronom, more efficient Cl^- excluder (clean box-whisker-plot). "Positive control" (abbreviated as "PC"), 120 mM Cl^- was supplemented into the nutrient solution. "+Cl^-", this abbreviation labels the chamber of the split-root device that was supplemented with 120 mM Cl^-. "-Cl^-", this abbreviation labels the chamber of the split-root device that was not supplemented with excess of Cl^-. "Negative control" (abbreviated as "NC"), no excess of Cl^- was supplemented into the nutrient solution. Small letters (regular font for P8589 and italic font for ES-Metronom) in (A) indicate significant differences by LSD test ($P < 0.05$) between different treatments in same genotypes. Asterisks in (B) represent significant differences (*, $P < 0.05$; **, $P < 0.01$) by T-test between P8589 and ES-Metronom. "nd", not detectable. Data are based on 4–8 biological replicates.

3.3. *No* Cl^- *translocation from leaf to root*

When aCl^--solution was brushed on the 5th fully developed leaf of a control plant, Cl^- concentrations of the same leaf increased from 8.8 mg g^{-1} DM in P8589 or 7.5 mg g^{-1} DM in ES-Metronom to 16.2 mg g^{-1} DM in P8589 ($P < 0.001$) or 16.9 mg g^{-1} DM in ES-Metronom ($P < 0.01$) (Fig. 6). A detailed analysis showed that foliar-applied Cl^- was mobile as concentration in blades ($P < 0.05$) and sheaths ($P < 0.01$) from older (non-foliar applied) leaves of the P8589 genotype increased as well. Such a difference was not detected in the ES-Metronom. Thus, it appears that mobility of Cl^- is better in P8589 than in ES-Metronom. Overall, the foliar applied Cl^- did not accumulate in the roots, as being revealed by a comparison between roots from control plants and plants that were foliar applied.

4. Discussion

4.1. *Restriction of* Cl^- *transfer from root to shoot*

The two maize (*Zea mays* L.) genotypes P8589 and ES-Metronom were able to keep shoot Cl^- concentration at a tolerable level even though 1 kg of the potting soil was supplemented with 757.1 mg Cl^- per kg dry soil. The toxic shoot concentration of 32.7 mg Cl^-g^{-1} DM as estimated for maize by Parker et al. (1985) was not exceeded (Fig. 2B) and there is no biomass reduction (Zhang et al., 2019). Attributable for this might be the ability to restrict acropetal transport of Cl^-, as indicated by a translocation factor <1 (Fig. 2A). This might explain why concentration of Cl^- is higher in roots than in shoot tissues (Fig. 2B). A TF smaller than 1, i.e. at 0,9, was also calculated for salt-tolerant

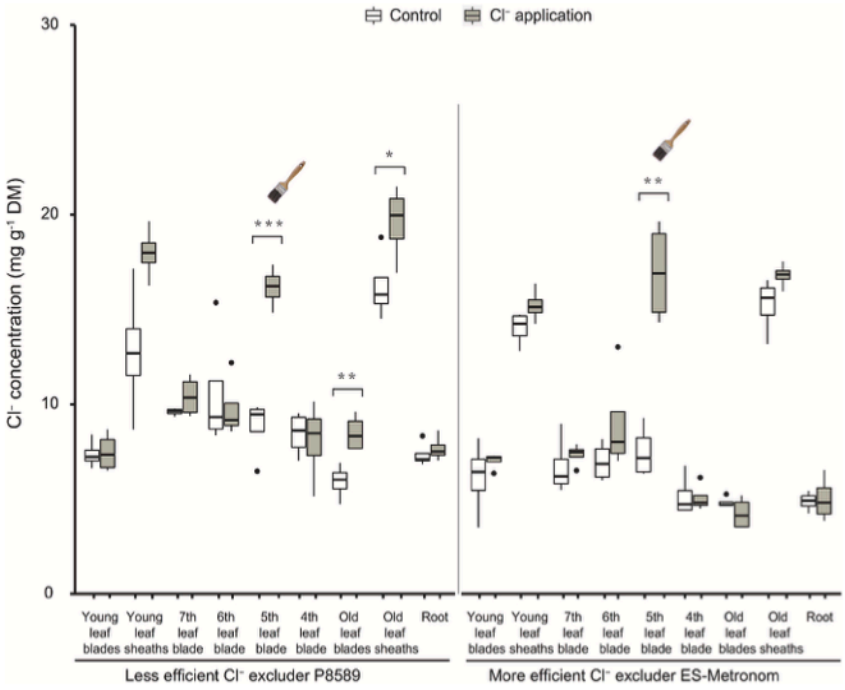

Fig. 6. Cl^- concentration in plant after Cl^- foliar application.
"Control", no foliar application of Cl^- and was filled by white color; "Cl^- application", with foliar application and was filled by gray color. Paintbrush shows leaf that received the exogenous Cl^--supply. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) by T-test between control- and foliar-applied plants in the same genotype. Data are based on 4 biological replicates.

beetroot (*Beta vulgaris* L. var. Crassa), a plant with halophyte ancestors (Zapata et al., 2017), or for the halophyte *Haloxylon recurvum* (TF 0.6) (Khan et al., 2000). The more salt-sensitive spinach (*Spinacia oleracea* L. cv. Boeing) had a TF greater than 1, i.e. of 1.25, when being stressed by salinity (Zapata et al., 2017). From a study on *Arabidopsis thaliana* it is known that reducing root to shoot transport of Cl^- is known to be an efficient means to reduce acropetal transport of Cl^- in (Li et al., 2016).

Moreover, a proportion of the Cl^- that is passed into the shoot is stored in sheaths of old leaves (Fig. 2B; small letters indicate differences between tissues within the same genotype). This could be a mechanism to avoid accumulation of Cl^- in both blades of old leaves and in younger shoot tissue, being a reason why maize is able to keep on growing under such a high Cl^- content. Notably, the less efficient Cl^- excluder P8589 seems to be less able to keep Cl^- concentration in the blades of old leaves as low as the more efficient Cl^--excluder ES-Metronom. Keeping Cl^- away from the site of primary photosynthesis and growing leaves is considered to be a mechanism to cope with Cl^- toxicity (Tavakkoli et al., 2010; Teakle and Tyerman, 2010).

In addition, we measured Cl^--concentration in different fractions of young leaves (the 7th and 8th leaves). While the sheaths of the young leaves showed the highest Cl^--concentration, concentration in blades was always lower. This substantiates that maize keeps Cl^- away from the photosynthetically active tissue. All blade segments of the less efficient Cl^- excluder P8589 (tip, center, edges, base) accumulated more Cl^- when compared to the more efficient excluder ES-Metronom (Fig. 3, indicated by asterisks). This corporates that the less efficient Cl^- excluder P8589 is starting having problems with keeping Cl^--concentrations as low as to the more efficient excluder ES-Metronom.

4.2. Cl^- storage in roots

Assuming that plants try to avoid accumulation of toxic compounds at the site of primary photosynthesis, roots might also be a place to store Cl^- under conditions of Cl^--salinity. Indication for this assumption is derived from one of the genotypes, namely P8589, in the split-root experiment. Roots of P8589 that grew in the chamber of the split-root system that was not supplemented with excess of Cl^- showed a Cl^- concentration of 11.6 ± 1.3 mg g^{-1} DM (mean ± SE). Of note, this concentration was significantly higher than in the roots of the negative control (3.5 ± 0.5 mg g^{-1} DM) (Fig. 4A). The only explanation for this discrepancy is that Cl^- ions that were taken up by the root that grew within the chamber of the split-root device that was supplemented with 120 mM ("+Cl^-") were translocated to roots of the same plant that grew within the other chamber where the nutrient solution was free of excess of Cl^- ("-Cl^-") (Fig. 4A). This phenomenon was confirmed under experimental condition where Cl^- uptake was reduced (Fig. 5A). Such conditions were implemented by inducing an uptake competition between Cl^- and NO_3^-, doing so by increasing NO_3^- concentration in the nutrient solution from 2 mM to 8 mM (Fig. 5A).

The second conclusion to be drawn by this study is that Cl^- can travel from roots that have been stressed by excess of Cl^- to roots that never experienced such a situation (Fig. 4A; Fig. 5A). We can exclude that this was due to a putative movement of ions within a water film that was attached outside of the roots and connected both chambers because in this case we would expect the Cl^--concentration in the nutrient solution to increase as well in the "-Cl^-"-chamber. This, however, was not the case (Figs. 4B and 5B). Storage of Cl^- in roots might be an effective means to dilute tissue concentration of Cl^- for avoiding that thresholds are exceeded. However, a few things remain to be elucidated. First, it is not known if this effectively occurs in nature when roots of the entire plant are in contact with a saline rooting media. Second, it remains unclear if such a Cl^- movement was result of a root-to-root translocation mechanism or result of a re-translocation of Cl^- from the shoot back to the root. In our work, we observed Cl^- movement in roots but not in both genotypes; thus, a generalization of this effect is not possible.

4.3. Re-translocation of Cl^- from shoot to root does not occur

In order to test whether a re-translocation of Cl^- from shoot to root exits, a third experiment was conducted. Leaves were brushed with a Cl^- solution. The fact that the foliar applied Cl^- traveled down into the old leaves was a strong indication that the foliar applied Cl^- was taken up, at least from the genotype P8589 (Fig. 6). Moreover, visual appearance of leaf edge necrosis proofed that the leaf application was an effective measure to induce salt stress (Fig. S1). In both genotypes, Cl^- was not re-translocated down to the roots (Fig. 6). This shows that there is no significant re-translocation of Cl^- from shoot to root in maize. By using ^{36}Cl as tracer, which is doubtless more sophisticated than our approach, Lessani and Marschner (1978) showed for maize that some Cl^- can be re-translocated from shoot to root, however, authors doubt that this is relevant under conditions of salinity because less than 3.5% were transferred into external medium. Munns and Fisher (1986) came also to the conclusion that such a mechanism may exist in barley, however, being without physiological significance. Based on the observation that basipetal re-translocation of Cl^- from shoot to root could not be observed, it appears that the accumulation of Cl^- in roots that grew in the chamber of the split-root device that did not contain excess of Cl^- was achieved by a transfer from root-to-root. A future task will be to gather understanding about the way how Cl^- moves from root to root. The symplastic route would require a protein facilitated cellular uptake. We assume that a transfer via the apoplastic route is also possible.

5. Conclusions

Maize seems to be able to grow in high chloride environments because it restricts acropetal transfer of Cl^-. The proportion of Cl^- reaching the shoot partly accumulates in sheaths of old leaves, thus away from the primary site of photosynthesis and growth. Within root tissues, it appears that Cl^- can travel from a root that experiences a high external Cl^- concentration to a root that grows in medium that is free of excess Cl^-. This might be helpful to dilute tissue concentration of Cl^-. Overall, the comparison between two maize genotypes that contrast in their ability to translocate Cl^- under conditions of Cl^--salinity shows that the maize germplasm is a diverse source of traits that could be mined in breeding efforts to engineer plants that perform better under condition of Cl^--salinity.

Author contributions

C-M. G and C. Z designed the research. XD. Z conducted the experiment and data analysis. C-M. G, XD. Z and C. Z wrote the manuscript. All authors have read and approved the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2020.06.036.

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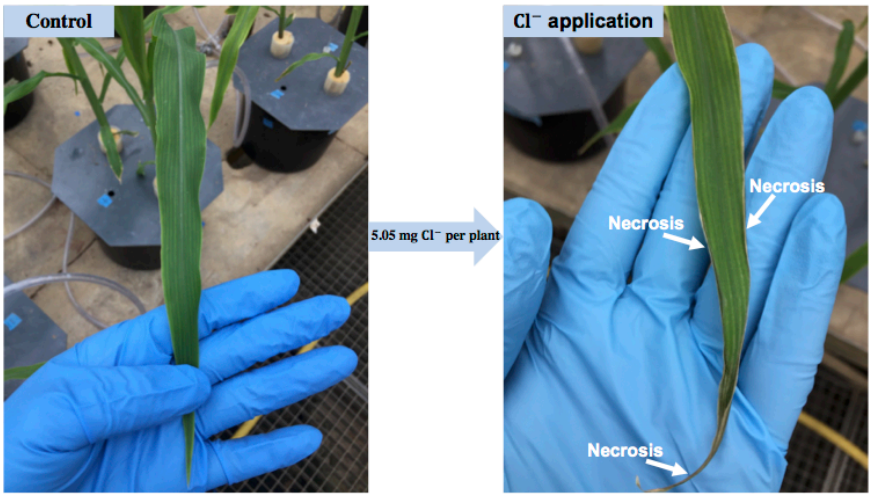
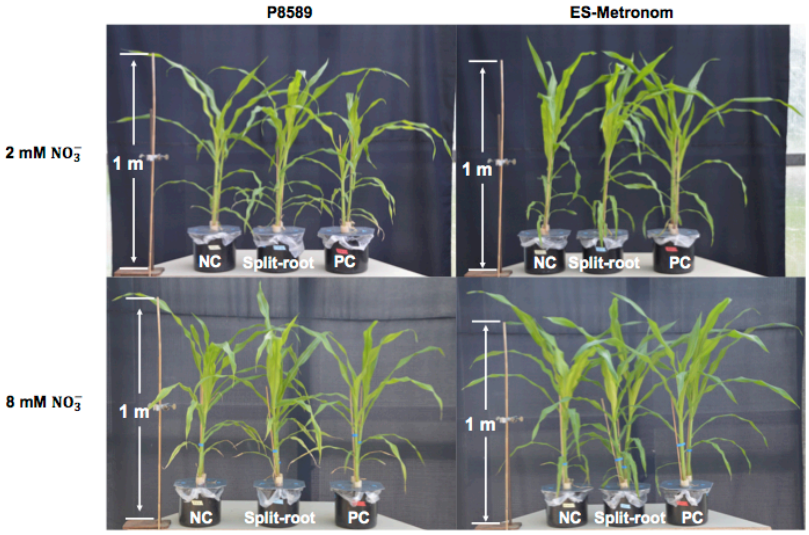
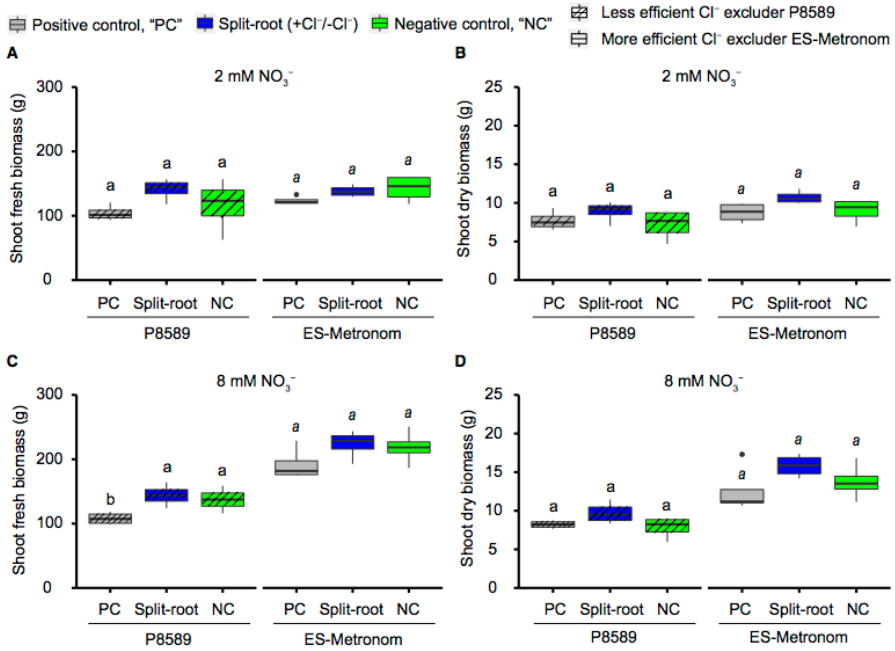
Supplemental figures



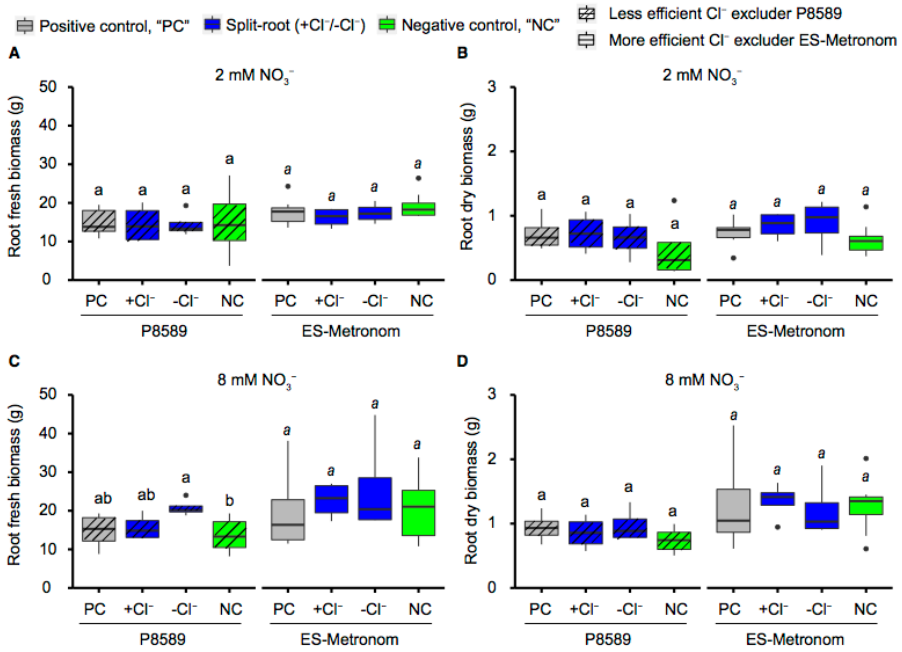
Supplemental Figure S1. Visual appearance of leaf that was exogenously treated with Cl^- via foliar application. 5.05 mg of Cl^- was brushed onto the leaf.



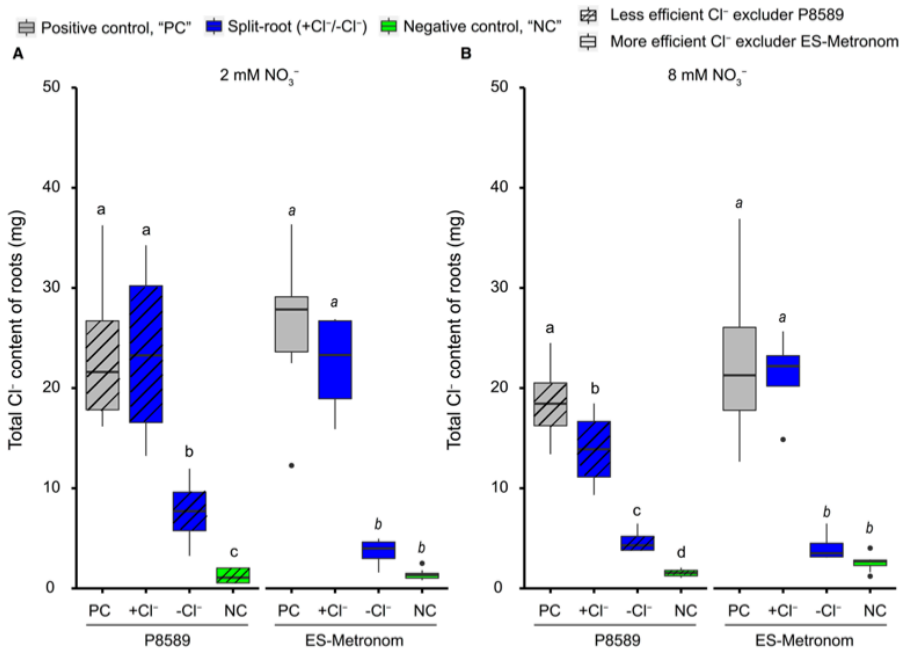
Supplemental Figure S2. Pictures of full-grown plants in split-root device under two levels of nitrate conditions. "Negative control" (abbreviated as "NC"), no excess of Cl^- was supplemented into the nutrient solution. "Split-root", one chamber was supplemented with 120 mM Cl^- while the other chamber was not supplemented with excess Cl^-. "Positive control" (abbreviated as "PC"), 120 mM Cl^- was supplemented into the nutrient solution.



Supplemental Figure S3. Shoot biomass of split-root device. Shoot fresh biomass under 2 mM NO_3^- condition (A), shoot dry biomass under 2 mM NO_3^- condition (B), Shoot fresh biomass under 8 mM NO_3^- condition (C), shoot dry biomass under 8 mM NO_3^- condition (D). P8589, less efficient Cl^- excluder (slashed box-whisker-plot); ES-Metronom, more efficient Cl^- excluder (clean box-whisker-plot). "Positive control" (abbreviated as "PC"), 120 mM Cl^- was supplemented into the nutrient solution. "Split-root", one chamber was supplemented with 120 mM Cl^- while the other chamber was not supplemented with excess Cl^-. "Negative control" (abbreviated as "NC"), no excess of Cl^- was supplemented into the nutrient solution. Small letters (regular font for P8589 and italic font for ES-Metronom) show significant differences by LSD-test between different treatments in the same genotype. Data are based on 4 biological replicates.



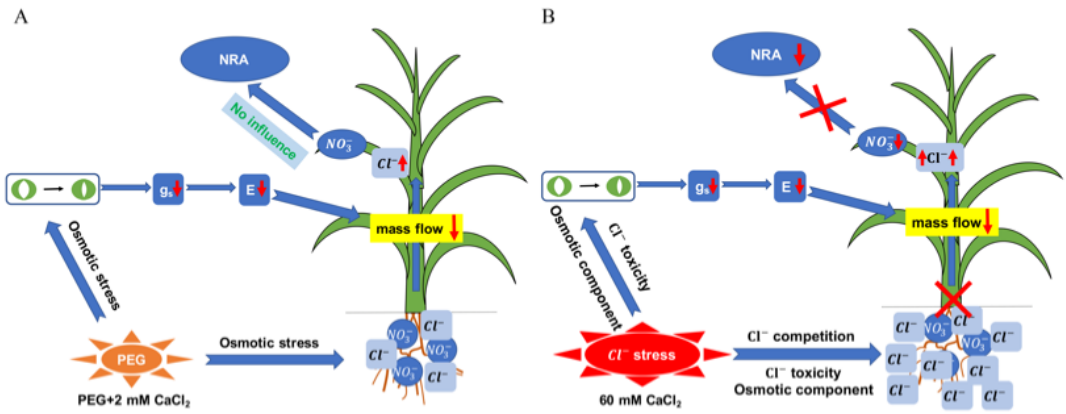
Supplemental Figure S4. Root biomass of split-root device. Root fresh biomass under 2 mM NO_3^- condition (A), root dry biomass under 2 mM NO_3^- condition (B), root fresh biomass under 8 mM NO_3^- condition (C), root dry biomass under 8 mM NO_3^- condition (D). P8589, less efficient Cl^- excluder (slashed box-whisker-plot); ES-Metronom, more efficient Cl^- excluder (clean box-whisker-plot). "Positive control" (abbreviated as "PC"), 120 mM Cl^- was supplemented into the nutrient solution. "Split-root", one chamber was supplemented with 120 mM Cl^- while the other chamber was not supplemented with excess Cl^-. "Negative control" (abbreviated as "NC"), no excess of Cl^- was supplemented into the nutrient solution. Small letters (regular font for P8589 and italic font for ES-Metronom) show significant differences by LSD-test between different treatments in the same genotype. Data are based on 4-8 biological replicates.



Supplemental Figure S5. Total Cl^- content of roots in split-root device under different nitrate levels. Total Cl^- content of roots under 2 mM NO_3^- condition (A), total Cl^- content of roots under 8 mM NO_3^- condition(B). P8589, less efficient Cl^- excluder (slashed box-whisker-plot); ES-Metronom, more efficient Cl^- excluder (clean box-whisker-plot). "Positive control" (abbreviated as "PC"), 120 mM Cl^- was supplemented into the nutrient solution. "+Cl^-", this abbreviation labels the chamber of the split-root device that was supplemented with 120 mM Cl^-. "-Cl^-", this abbreviation labels the chamber of the split-root device that was not supplemented with excess of Cl^-. "Negative control" (abbreviated as "NC"), no excess of Cl^- was supplemented into the nutrient solution. Small letters (regular font for P8589 and italic font for ES-Metronom) indicate significant differences by pair-wise T-test ($P<0.05$) between different treatments in same genotypes. Data are based on 4-8 biological replicates.

6. Chapter III

Antagonism of chloride and nitrate inhibits nitrate reductase activity in chloride-stressed maize



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ORIGINAL PAPER

Antagonism of chloride and nitrate inhibits nitrate reductase activity in chloride-stressed maize

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Abstract
Chloride (Cl^-) is required for photosynthesis and regulates osmotic balance. However, excess Cl^- application negatively interacts with nitrate (NO_3^-) uptake, although its effect on NO_3^- metabolism remains unclear. The aim was to test whether Cl^- stress disturbs nitrate reductase activity (NRA). A maize variety (*Zea mays* L. cv. LG 30215) was hydroponically cultured in a greenhouse under the following conditions: control (2 mM $CaCl_2$), moderate Cl^- (10 mM $CaCl_2$), high Cl^- (60 mM $CaCl_2$). To substantiate the effect of Cl^- stress further, an osmotic stress with lower intensity was induced by 60 g polyethylene glycol (PEG) 6000 L^{-1} + 2 mM $CaCl_2$), which was 57% of the osmotic pressure being produced by 60 mM $CaCl_2$. Results show that high Cl^- and PEG-induced osmotic stress significantly reduced shoot biomass, stomatal conductance and transpiration rate, but NRA was only decreased by high Cl^- stress. The interference of NRA in chloride-stressed maize is supposed to be primarily caused by the antagonistic uptake of Cl^- and NO_3^-.

Keywords Chloride stress · Nitrate · Osmotic stress · Photosynthesis

Introduction

Salt stress caused by, for example, NaCl is a serious abiotic threat that imposes negative impacts on crop yield and the quality of plant products (Abdelaal et al. 2020; Naeem et al. 2020; Zörb et al. 2019). The unfavorable effects on plant growth are attributed to either osmotic stress or ion toxicity (Munns and Tester 2008). Chloride (Cl^-) is the anion of NaCl and has multiple functions in plants (Geilfus 2018a; Wege et al. 2017). Being a micronutrient (µM range), Cl^- acts as a dominant functional element involved in the oxygen-evolving complex of PSII of photosynthesis (Kawakami et al. 2009) and the regulation of enzymatic activity such as asparagine synthase (Rognes 1980) and V-ATPase in the tonoplast (Geilfus 2018b). When the Cl^- concentration lies on a macronutrient level (low millimolar range), it can be beneficial for enhancing water-use efficiency and net photosynthesis rate (A_N) (Rosales et al. 2020) and, as a consequence, for promoting plant growth and biomass production in citrus (Brumos et al. 2010) and tobacco plants (Franco-Navarro et al. 2019). The enhanced plant growth gives indication that Cl^- might also increase nitrogen use efficiency (NUE) (Rosales et al. 2020). However, if Cl^- application exceeds favorable doses, plants may suffer from disturbed ion homeostasis, photosynthetic disorders, reduced biomass, or even cell death (Geilfus 2018b; Wege et al. 2017).

Nitrate reductase (NR) is a substrate-induced enzyme that catalyzes the conversion of NO_3^- to NO_2^- and the production of nitric oxide (NO) (Chamizo-Ampudia et al. 2017; Crawford and Glass 1998; He et al. 2017). NR plays a pivotal role in massive biological processes such as photosynthesis, molecular biology in chloroplast, starch synthesis and metabolism, and nutrient availability and metabolism by regulating not only the availability of nitrite for nitrogen assimilation but also the homeostasis of the signaling

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molecule NO (Chamizo-Ampudia et al. 2017). As a substrate, the NO_3^- concentration has been well documented as positively correlating with nitrate reductase activity (NRA) (Mengel et al. 1983; Hütsch et al. 2016); however, this correlation can differ depending on the analyzed tissue (Mengel et al. 1983). For instance, the correlation of NO_3^- and NRA exhibits a saturation curve and a sigmoidal curve in maize leaves and roots, respectively (Mengel et al. 1983). Hence, NRA is inevitably regulated by the availability of the resource NO_3^-. Stressful conditions have also been reported to decrease NRA in a dose-dependent manner, as shown with regard to water shortage (Larsson et al. 1989; Munjal et al. 1997) and NaCl stress (Botella et al. 1993; Khan et al. 1995) in various plant species. Reduced NRA is assumed to result from stress-related disturbances of NR expression (Lu et al. 1992) and the conversion of the enzyme to an inactivated form (Munjal et al. 1997).

The monovalent macronutrient anion NO_3^- possesses similar physical properties to Cl^- (Wege et al. 2017). This similarity of physical properties leads to an antagonistic relationship in the uptake between these two anions. Under saline conditions, excess Cl^- may reduce the availability of NO_3^- as a substrate that supports the activity of NR. Moreover, the transport of NO_3^- between the vacuole and cytoplasm is regulated by chloride channels such as AtCLCa (Zhang et al. 2017). The biosynthesis and activity of NR are also thought to be influenced by Cl^-, but no experiments have been conducted to substantiate this speculation (Flores et al. 2000). Nevertheless, a direct causation between Cl^- and the inhibition of NRA remains elusive in many crops such as maize. This might be because the sodium component of NaCl salinity has received more attention over the last few decades.

Maize, as a moderately salt (NaCl)-sensitive crop (Farooq et al. 2015), has recently been established to tolerate Cl^- stress moderately in our previous work (Zhang et al. 2019). From this previous work, a mildly tolerant genotype was selected to answer the following questions: (i) does Cl^- stress or osmotic stress influence maize growth and photosynthesis; (ii) does Cl^- stress or osmotic stress influence on NRA in leaf tissues?

Material and methods

Plant material and cultivation

The maize variety (*Zea mays* L. cv. LG30215 supplied by LG c/o Limagrain GmbH), mildly tolerant to Cl^- stress (Zhang et al. 2019), was hydroponically cultured in 5-l pots (two plants per pot) with four biological replicates for each of the treatments, respectively, in the greenhouse, which means eight plants in total under each condition (Fig. 1a). Grains were germinated for 13 days in sand and transferred to hydroponic solutions at the two-leaf stage. The initial nutrient solution was one third of full strength and then was stepwise increased to two thirds and final full strength every second day. The full nutrient recipe (Richter et al. 2015; Zörb et al. 2015) contains the macronutrients $Ca(NO_3)_2$ 0.66 mM, NH_4NO_3 1.33 mM, KH_2PO_4 0.2 mM, K_2SO_4 1.0 mM, $MgSO_4$ 0.5 mM, $CaCl_2$ 2 mM, and Fe-EDTA 0.2 mM and the micronutrients H_3BO_3 5.0 µM, $MnSO_4$ 2 µM, $ZnSO_4$ 0.5 µM, $CuSO_4$ 0.3 µM, and $(NH_4)_2Mo_7O_{24}$ 0.01 µM. Hydroponic solutions were changed twice a week. The initial pH value was approximately 6.1, which could be kept constant without any precipitation for 4 days.

Maize seedlings were treated under the following conditions: control (2 mM $CaCl_2$), moderate Cl^- (10 mM $CaCl_2$), high Cl^-(60 mM $CaCl_2$), and osmotic stress (60 g L^{-1} PEG 6000 (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) + 2 mM $CaCl_2$). The reason to define 10 mM $CaCl_2$ as moderate stress and 60 mM $CaCl_2$ as high stress is that plant heights by both stresses were always lower than the control and the height of plants treated by 10 mM $CaCl_2$ were constantly higher than those of 60 mM $CaCl_2$ (Fig. S1). In order to substantiate the effects of Cl^-, 2 mM $CaCl_2$ was added to PEG-induced osmotic stress, as in the control. PEG-induced osmotic pressure (130.1 mOsm kg^{-1}) was only 57% of that produced by 60 mM $CaCl_2$ (228.9 mOsm kg^{-1}). The effect of Ca^{2+} on maize growth can be excluded, because the Ca^{2+} concentration in young leaves was 10 mg g^{-1} DM under 60 mM $CaCl_2$, which is located in the reported optimal range (2.1 to 16 mg g^{-1} DM) for maize leaves (Gaj et al. 2018; Johnston and Dowbenko 2004). Thus, the detrimental effects of increased Ca^{2+} concentrations were considered to be marginal. The treatments were firstly applied to the plants in nutrient solutions, with half strength at the 7th day after transfer and were afterwards supplemented to reach full strength at the 9th day. The youngest fully developed leaf blade was selected at the first day of treatment. At the 28th day after transfer of the plants into the hydroponic solution, the whole plant was harvested and separated into four fractions (Fig. 1b): young part, fully developed leaf blade, old part, and root. Among them, the young part and old part were a mixture of corresponding leaf blades and leaf sheaths.

Fresh weight and dry weight

The fresh weight of each plant fraction was immediately obtained at harvest. The dry weight was measured after the leaves had been dried at 60 °C for 4 days in a ventilated oven.

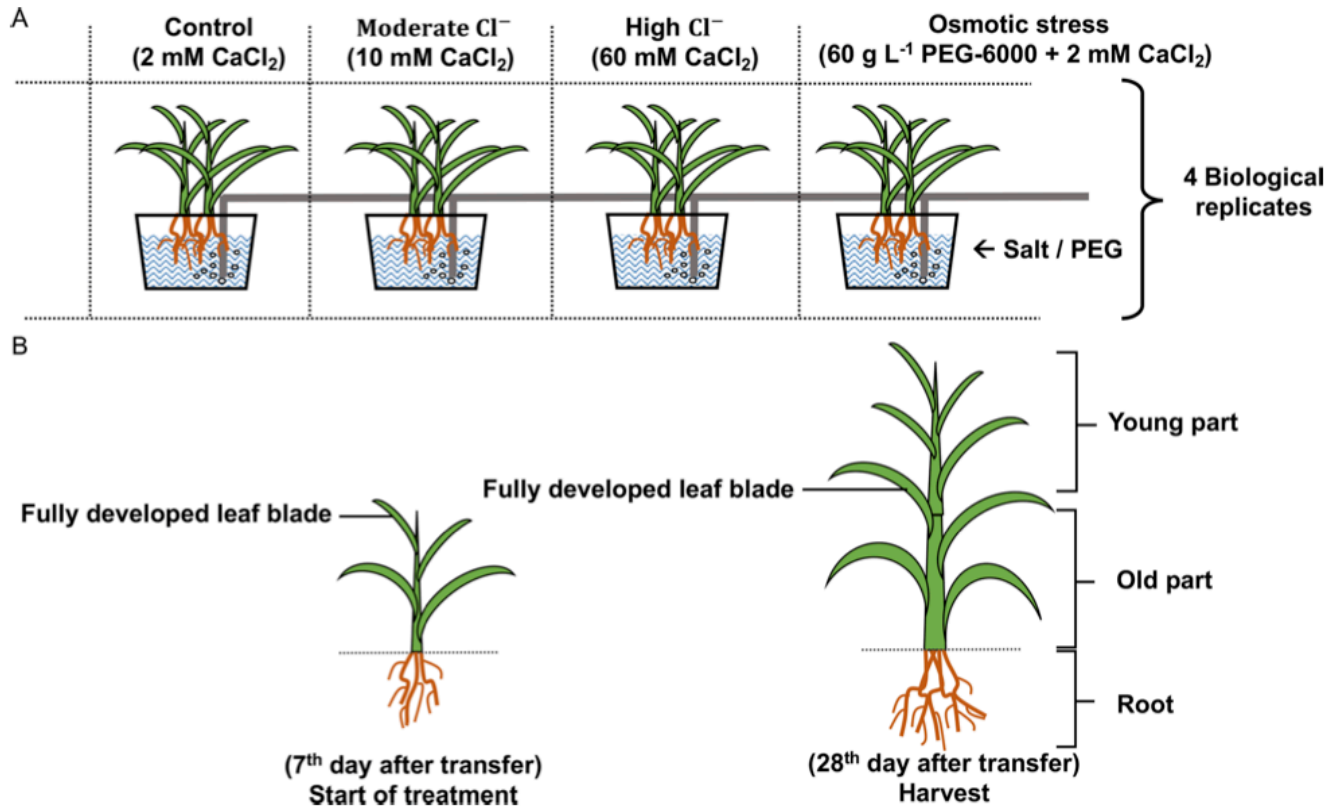

Fig. 1 Experimental design. **a** Experimental set-up of maize plants cultured in hydroponic solution, **b** diagrams of treatment application and harvest

Chloride concentration

Dry materials of each fraction were uniformly ground into fine powder by a mill (Retsch ZM1) equipped with a 0.5 mm sieve mesh. Plant tissue powder (200 mg) was dissolved in 10 mL dH_2O in glass tubes. After being vortexed, the test tubes were covered with glass beads to prevent the escape of water, heated at 80 °C for 15 min, and then quickly cooled for 7 min in the ice bath. The cooled extracts were filtered through pleated filters in 15 mL plastic centrifuge tubes. The Cl^- concentration was determined by using a chloride meter (6610, Eppendorf AG, Hamburg, Germany) (Zhang et al. 2019). The extract solution (100 µL) was pipetted into the titration solution (a mixture of 1 mL gelatin plus indicator solution, Biorapid GmbH, Freiburg, Germany) and 15 mL acid buffer (6.4 mL L^{-1} 65% nitric acid and 57.6 mL L^{-1} 100% acetic acid). Each sample was measured in three technical replicates.

Nitrate reductase activity

The activity of nitrate reductase (EC 1.7.1.1) was determined according to the method of Hageman and Hucklesby (Hageman and Hucklesby 1971) with slight modification. Frozen leaf material (− 80° C) was ground in liquid nitrogen, and 0.2 g of this leaf powder was weighed into 2 mL reaction vessels. After the addition of 1 mL digestion buffer (100 mM TRIS / HCl pH 8.0, 1 mM EDTA, 10 mM cysteine) with thorough vortexing, centrifugation was carried out at 4 °C for 10 min at 20,000×*g*. The supernatant was pipetted into a new reaction vessel and placed on ice. For each sample, two reaction tubes were prepared according to the following scheme: 400 µL protein extract and 600 µL reaction buffer (100 mM KNO_3; 1 mM $NADH^+$ H^+; 1.08 mM K_2HPO_4; 1.47 mM Na_2HPO_4) were mixed (batch reaction). To one of the tubes (reference tube), 200 µL batch stopper (zinc acetate dehydrate; 1 M $C_4H_6O_4Zn•2H_2O$) was immediately added in order to estimate the initial concentration of NO_2^- in leaf samples. This reference tube was subsequently incubated on ice for 30 min. Meanwhile, the second tube with exclusive reaction buffer was also incubated for 30 min at 30 °C before the batch stopper (zinc acetate, 200 µL) was added to stop the reaction. The reaction mixtures were then centrifuged at 20 °C for 2 min at 20,000×*g*. Aliquots of 500 µL supernatant from these two tubes were each subsequently pipetted into empty tubes, followed by the addition of 500 µL sulfanilamide (25% HCl; 10 mg L^{-1} and 500 µL naphthyl reagent (N-(1-naphthyl) ethylene diamine dihydrochloride; 0.2 mg L^{-1}) and allowed to stand for 15 min at room temperature for color development. For the blank and the calibration

curve, a similar mixture was prepared in each case with 500 µL ddH_2O or nitrite standards (0.05 mM, 0.1 mM, 0.5 mM, 1 mM, 5 mM, and 10 mM KNO_2) replacing the supernatant. Finally, the absorbance at 540 nm was measured against the blank value with the multimode reader TriStar S LB 942 (Berthold Technologies, Bad Wildbad, Germany). The NRA is described as the weight of the formed reaction product (NO_2^-) per tissue fresh mass and time (µmol NO_2^- g^{-1} FM h^{-1}). Three technical replicates were performed.

Nitrate concentration

NO_3^- concentration was determined by a UV/Vis spectrophotometer (SPECORD®50 PLUS, Analytik Jena AG, Germany) via the nitration of salicylic acid (Xu et al. 2016) with a downscaling modification. In brief, liquid-ground maize leaf tissue (100 mg) was dissolved in 1 mL dd H_2O and heated at 90 °C for 30 min. After being cooled to room temperature, samples were centrifuged at 14,000×*g* for 10 min. The extracted supernatant (20 µL) was transferred to new tubes in parallel with the same amount of standard solution and the blank reference. Salicylic acid-sulfuric acid (80 µL) was added to the supernatant, completely mixed, and then incubated at room temperature for 20 min. Subsequently, NaOH (1.9 mL; 80 mg L^{-1}) was added to the tubes in order to generate a high alkaline environment in which samples were able fully to develop the color. These reaction tubes had to be cooled at room temperature for 30 min, since the concentrated NaOH addition induced the release of heat. The absorbance of all samples was measured at the wavelength of 410 nm with the control as reference. Each sample was technically repeated three times.

Estimated chlorophyll concentration

The chlorophyll content of the fully developed leaf blade was estimated by SPAD meter (Model No. 502, Konica Minolta) (Ebert et al. 2002). For the sake of accuracy, leafy cubes from various positions on the fully developed leaf blade were randomly chosen on both sides of the central vein. The final value was averaged over three measurements.

Stomatal conductance, transpiration rate, and net photosynthetic assimilation

The fully developed leaf blade (Fig. 1b) was used for monitoring stomatal conductance (g_s), transpiration rate (E) and net photosynthetic rate (A_N) with a LCi Portable Photosynthesis System installed with a broad chamber (ADC BioScientific Ltd) (Zhang et al. 2019). Illumination of the chamber mimicked natural sunlight ranging from 150 to 1200 µmol m^{-2} s^{-1} per leaf area. In the tested leaf blade, CO_2 diffusion into leaf chamber was 400 ppm, and H_2O flux was 0.17 µmol m^{-2} s^{-1}.

Statistical analysis

Data of all four biological replicates are used to make whisker-box plots. The significant difference between the control and other treatments was evaluated at the probability of 0.05 and 0.01 levels by two factor ANOVA and the LSD test with R studio 1.1.456 (R studio Inc, Boston, USA) with library (agricolae) (De Mendiburu 2017). Principal component analysis (PCA) was analyzed and plotted by R studio 1.1.456 (R studio Inc, Boston, USA) with function "prcomp" (Venables and Ripley 2002) and library (ggbiplot) (Vu 2016) on a basis of 16 parameters with biological replicates measured as above. The Cl^- concentration, osmolality, NRA, and NO_3^- concentration were measured within three technical replicates, respectively.

Results

Plant biomass and plant height

All plant organs showed significantly reduced fresh mass under high Cl^- (60 mM $CaCl_2$) or osmotic stress when compared with the control (Fig. 2a). No significant difference in fresh weight could be observed between moderate Cl^- treatment and the control, except for the total shoot (Fig. 2a). In contrast, negative effects of high Cl^- and osmotic treatment on dry weight were present in young tissue, old tissue, and shoots but not in roots (Fig. 2b). With the time of exposure to treatments increased, plant heights by 10 mM $CaCl_2$, 60 mM $CaCl_2$, and PEG-induced osmotic stress were constantly lower than the control. Among these treatments, 10 mM $CaCl_2$ led to the highest plant height while osmotic stressed-plant was the lowest (Fig. S1).

Chloride concentration of all fractions, nitrate reductase activity and nitrate concentration in the fully developed leaf blade

Cl^- concentrations of all plant tissues significantly rose with increasing Cl^- application, except for young tissue under moderate Cl^- treatment (Fig. 3a). Maximum of Cl^- concentrations, as expected, appeared under high Cl^- treatment. The Cl^- concentration of all fractions under osmotic treatment was similar to that of the control, except for the fully developed leaf blade, which was slightly higher (Fig. 3a). This increase is attributable to

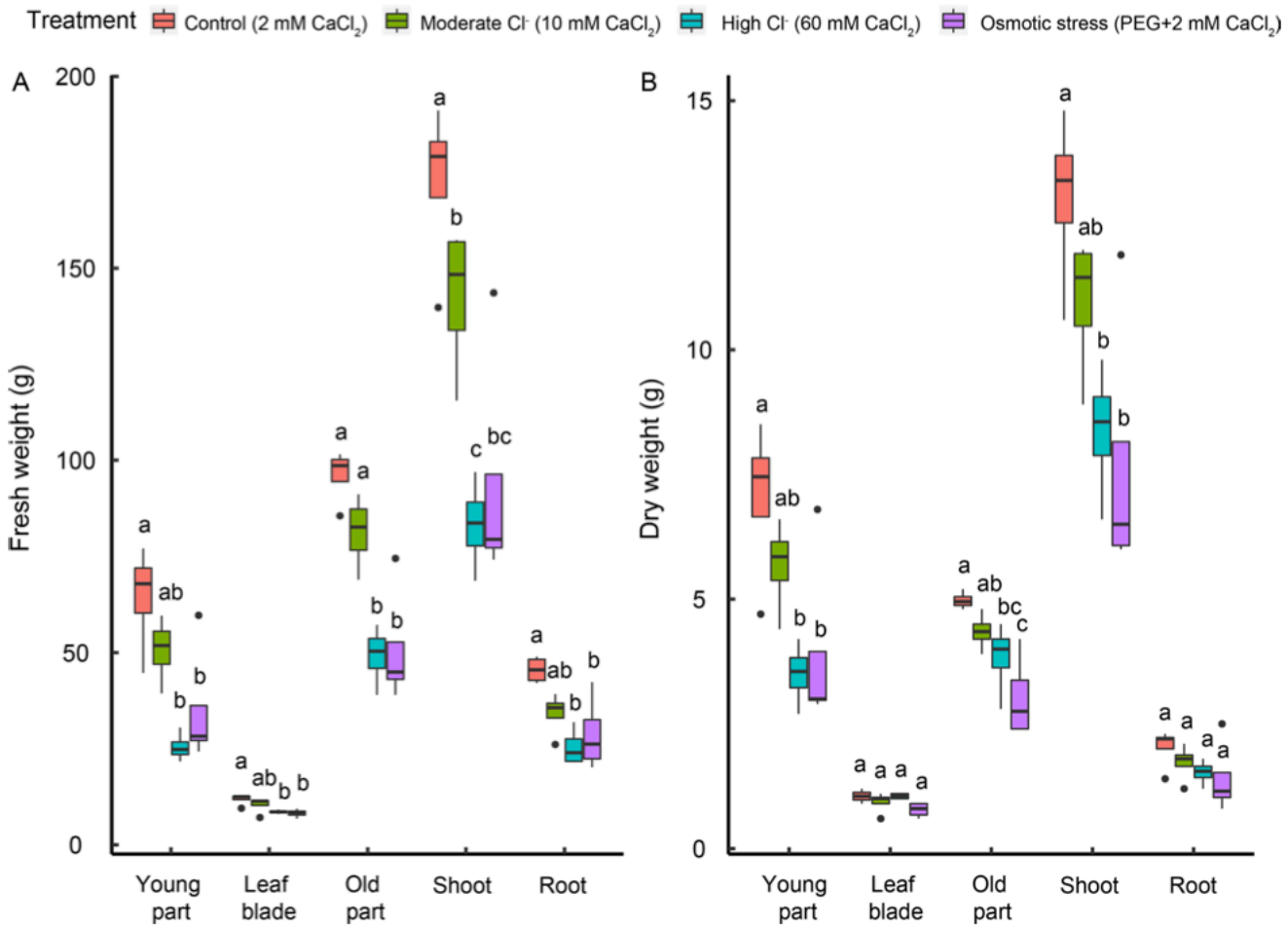

Fig. 2 Fresh and dry biomass of various fractions in maize. **a** fresh weight per pot, **b** dry weight per pot. Small letters represent significant differences ($P < 0.05$, LSD test) in one tissue group under the different treatments (n = 4). Shoot represents all above-ground parts, i.e., a mathematic sum of the young part, fully developed leaf blade, and old part

the fact that the treatment termed "osmotic treatment" also included 2 mM $CaCl_2$. Under moderate to high Cl^- application (10 mM to 60 mM $CaCl_2$), more chloride accumulated in old tissues and roots than in younger tissues (Fig. 3a).

NR exhibited the greatest activity under control conditions (0.37 µmol NO_2^- g^{-1} FM h^{-1}) and moderate Cl^- treatment (0.43 µmol NO_2^- g^{-1} FM h^{-1}), but the lowest activity appeared under high Cl^- treatment (0.17 µmol NO_2^- g^{-1} FM h^{-1}) (Fig. 3b). No difference was detected between PEG-induced osmotic treatment and control (Fig. 3b).

In accordance with NR, NO_3^- concentration showed the similar pattern. It is clear to see that NO_3^- concentration (0.0075 mmol g^{-1} FM) was the lowest under high Cl^- treatment and the maximum (0.0265 mmol g^{-1} FM) was observed in moderate Cl^- treatment (Fig. 3c). In comparison with control, osmotic treatment (plus 2 mM $CaCl_2$) was associated with the same concentration of NO_3^- (Fig. 3c).

Estimated chlorophyll concentration, stomatal conductance, transpiration rate, and net photosynthetic rate in the fully developed leaf blade

The chlorophyll concentration per unit of leaf area, as estimated by SPAD readings, gradually increased from 6 to 13 days after treatment (DAT) and afterwards reached a constant level at 18 and 19 DAT in control, moderate Cl^- and high Cl^- (Fig. 4a). Under the condition of osmotic stress, the chlorophyll concentration always kept constant among the whole period and was also significantly lower than other conditions at 13, 18, and 19 DAT (Fig. 4a). Notably, there were no differences in SPAD readings between different Cl^- applications regardless of how long the plants were treated (Fig. 4a).

High Cl^- stress and osmotic stress significantly decreased g_s and E (Fig. 4b and c) but did not reduce A_N

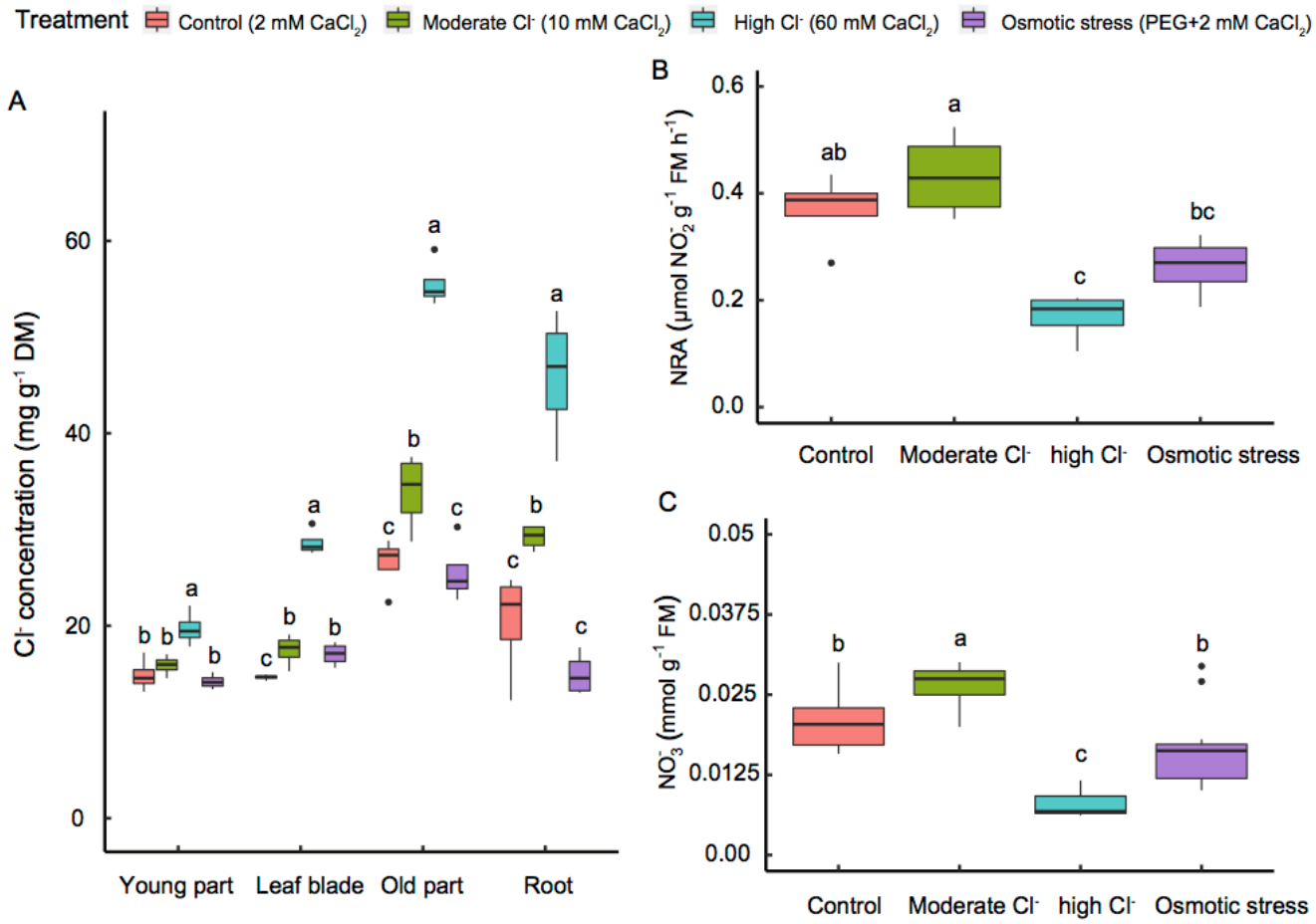

Fig. 3 Chloride concentration of all fractions, nitrate reductase activity and nitrate concentration in the fully developed leaf blade. **a** Cl^- concentration of all fractions, **b** nitrate reductase activity (NRA) in the fully developed leaf blade, **c** NO_3^- concentration in the fully developed leaf blade. Small letters represent significant differences ($P < 0.05$, LSD test) in one tissue group under the different treatments (Cl^- concentration and NRA, n = 4; NO_3^- concentration, n = 12)

(Fig. 4d) and intercellular CO_2 concentration (Fig. S2) in comparison with the control.

Principal component analysis

In principal component analysis (PCA) (Fig. 5), the high Cl^- treatment and osmotic treatment were primarily found on the right side of the first and fourth quadrants of the middle axis, respectively, whereas the control and moderate Cl^- treatment generally lay together in the second and third quadrants. All 15 physiological variables in response to the various Cl^- applications were comprehensively analyzed, and a large part of the variability could be explained by the first two principal components (PC1 44.6% and PC2 18.4%). In detail, the osmolality (data shown in Fig. S3) of all fractions was allocated in the high Cl^- cluster. The fresh and dry biomass of shoots and roots and the SPAD were in the upper left quadrant. Moreover, the gas exchange variables (g_s, E and A_N), NO_3^- metabolism parameters (NRA and NO_3^- concentration), and ion leakage (data shown in Fig. S4) clustered in the lower left quadrant.

Discussion

Effects of Cl^- application and osmotic stress on biomass

The present experiments indicate that Cl^- has no direct ionic toxicity on shoot biomass, in which the osmotic component induced by excessive Cl^- is responsible for the biomass reduction. This is verified by the biomass data of PEG-induced osmotic stress (Fig. 2), in which a lower osmotic pressure (only 57% of that produced by high Cl^-) results in a shoot biomass reduction as similar as high Cl^- stress for 19 days after treatment. Besides, the close negative correlation between the biomass of whole plants and PEG-induced osmotic stress indicated by the PCA pattern (arrows in opposite directions,

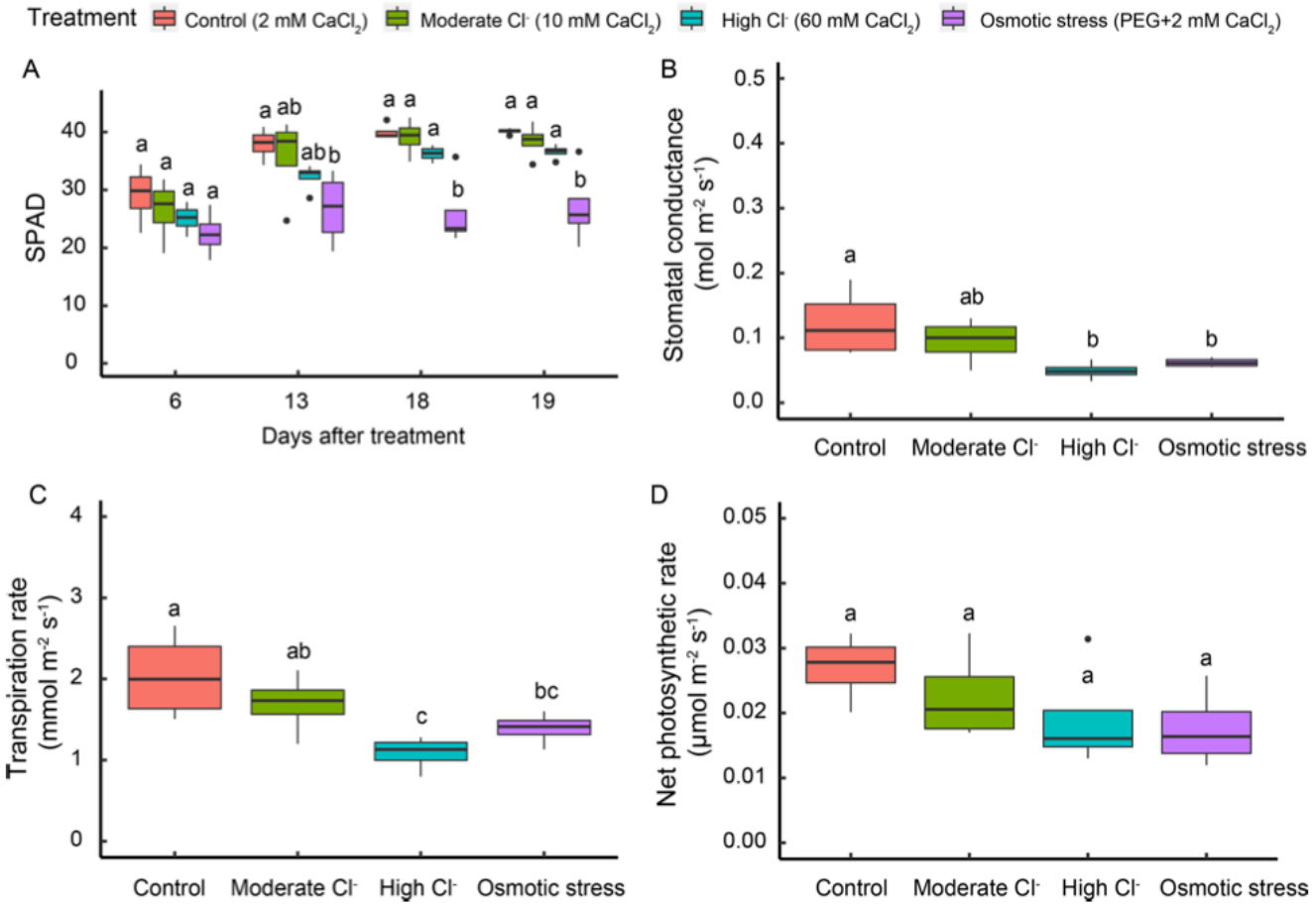

Fig. 4 Estimated chlorophyll concentration, stomatal conductance, transpiration rate, and net photosynthetic rate in the fully developed leaf blade. **a** Estimated chlorophyll concentration as indicated by SPAD, **b** stomatal conductance, **c** transpiration rate and **d** net photosynthetic rate. In subfigure A, small letters represent significant differences ($P<0.05$, LSD test) under the different treatments at each time point after treatment ($n=4$). In subfigures B, C and D, average values of the 6th, 13th, 18th, and 19th days after the treatment are regarded as raw data for whisker-box plotting. Small letters represent significant differences ($P<0.05$, LSD test) in stomatal conductance, transpiration rate, and net photosynthetic rate under the different treatments ($n=4$)

Fig. 5) suggest that osmotic stress plays a more vital role in the reduction of plant biomass. Furthermore, the similar visual appearance of small necrotic symptoms on the older leaves (Fig. S5) is observed under both high Cl^- stress and the PEG-induced osmotic treatment. The necrotic symptoms of old leaves are believed to be caused by osmotic stress, which was also found in NaCl stressed maize seedlings (Cramer et al. 1994; De Costa et al. 2007). The comparisons of shoot biomass (Fig. 2) and necrotic symptoms (Fig. S5) between PEG-induced osmotic treatment and control further proves that the osmotic-stress-induced growth inhibition or leaf necrosis in maize is not specific to Cl^-.

Context of Cl^- and estimated chlorophyll concentration, stomatal conductance, transpiration rate, and photosynthesis rate

Osmotic stress is a serious threat to chlorophyll concentration indicated by SPAD; indeed, it caused a decrease of 32.6% under PEG-induced osmotic treatment (Fig. 4a) and a clear negative correlation between SPAD and osmotic stress cluster by PCA plots (Fig. 5). This is in agreement with the observation that water stress can reduce the chlorophyll concentration up to 40% without adversely affecting photosynthesis at mid-day in maize (Sanchez et al. 1983).

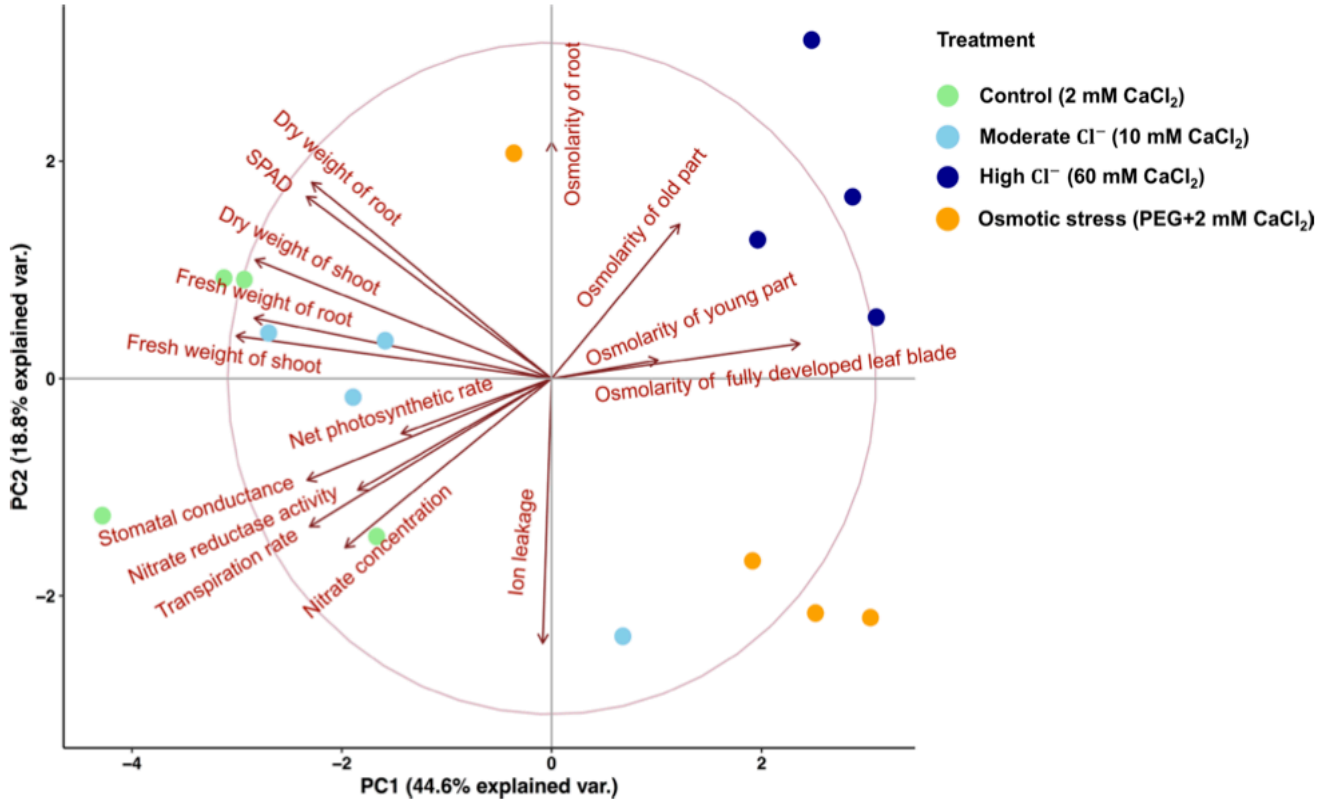

Fig. 5 Principal component analysis of 15 variables in maize responding to Cl^- application or osmotic treatment

In parallel, the uncompromised photosynthesis was also observed in the present experiment (Fig. 4d). Interestingly, chlorophyll reduction did not appear in high Cl^- application with higher osmotic stress (Fig. 4a). It could be resulted from the osmotic adjustment of Cl^-, in which the leaf blade had a considerably higher Cl^- concentration under 60 mM $CaCl_2$ than PEG-induced osmotic stress (Fig. 3a). In addition, there was no significant difference in SPAD readings between varied Cl^- doses at the whole treatment period. Therefore, reductions of chlorophyll concentration in maize result from osmotic stress rather than from Cl^- ionic toxicity.

The reduced stomatal conductance (g_s) (Fig. 4b) decreased E (Fig. 4c) but did not disturb A_N (Fig. 4d) under high Cl^- stress and PEG-induced osmotic stress. One reason might be that the stomatal regulation predominantly minimizes water loss while only marginally inhibiting CO_2 assimilation (Farquhar and Sharkey 1982). This can be evidenced by the data (Fig. 4b and Fig. S2), which showed that reduced g_s did not influence intercellular CO_2 concentration by high Cl^- stress and osmotic stress.

Inhibitory effects of Cl^- stress on NRA in maize

The negative correlation of NO_3^- metabolism variables (NO_3^- concentration and NRA) and gas exchange parameters (g_s, E, and A_N) with Cl^- stress was stronger than that with osmotic stress, as shown by the PCA results (Fig. 5). Therefore, the correlation differences indicate that the mechanism of NO_3^- metabolic interference between osmotic stress and Cl^- stress is different (as shown by proposed mechanisms in Fig. 6).

Under PEG-induced osmotic stress (plus 2 mM $CaCl_2$), the osmotic imbalance caused more Cl^- to enter into the leaf cells (Figs. 3a and 6a) probably because of Cl^- as a `cheap´ osmoticum (Wege et al. 2017). Apart from this, Cl^- is also thought to liberate (by means of substitution) other osmotica such as proline, sucrose, malate, K^+, and NO_3^- for use in other functions (Flowers 1988). The osmotic adjustment of Cl^- is beneficial to help plants adapt the osmotic stress. Although PEG-induced osmotic stress significantly reduced E (Figs. 4c and 6a), the decreased mass flow did not induce a reduction of NO_3^- concentration in the fully leaf blade as well as NRA (Figs. 3b, c and 6a).

The addition of excess Cl^- has considerably reduced NO_3^- concentration in the fully developed leaf blade, and thereby decreasing NRA (Figs. 3 and 6b). It arises a question how the overdose of external Cl^- results in a considerable reduction of NO_3^- concentration in the distant leaves via the competitive uptake with NO_3^- or the reduced NO_3^- translocation by decreased mass flow or both. The antagonistic uptake between Cl^- and NO_3^- has been well documented, because these two monovalent anions share a NO_3^- transporter *Zm*NPF6.4, which is a root-located transmembrane protein

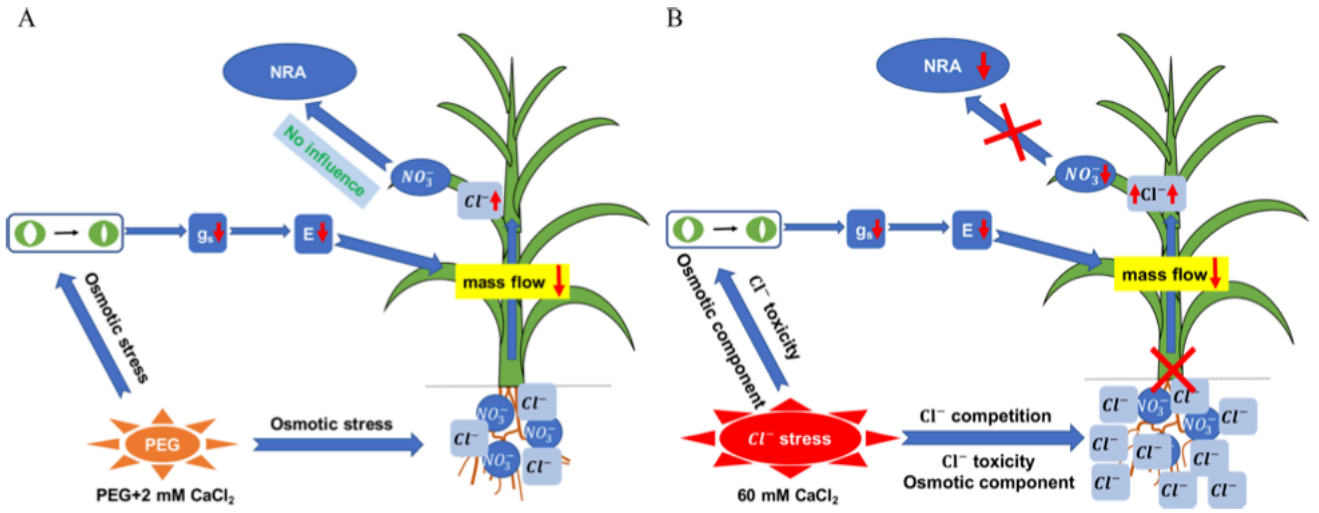

Fig. 6 Proposed mechanisms of NRA interference by Cl^- stress or osmotic stress in maize. **a** PEG-induced osmotic stress + 2 mM $CaCl_2$, **b** Cl^- stress (60 mM $CaCl_2$). Abbreviations are as following: *Cl*$^-$ chloride, *NO*$^-_3$ nitrate, *NRA* nitrate reductase activity, g_s stomatal conductance, *E* transpiration rate, "↑", an increase of the corresponding parameter, "↓", a decrease of the corresponding parameter, "X" the breakdown of the corresponding biological process

(Wen et al. 2017). Wen et al. (2017) found that an increase of external Cl^- concentration from 0 to 10 mM could facilitate Cl^- uptake of *Zm*NPF6.4. It explains the competition to NO_3^- uptake. Besides, the effect of reduced NO_3^- translocation via mass flow could be excluded or at least very tiny, although the data of NO_3^- xylem translocation was not measured at the present work. It can be well evidenced by transpiration rate data (Fig. 4c), because PEG-induced osmotic stress and 60 mM $CaCl_2$ stress caused the similar reduction of E, but the decreased mass flow indicated by E did not show a negative effect on leaf NO_3^- concentration under PEG-induced osmotic stress (Figs. 3c and 4c). Therefore, it is reasonably believed that the competitive uptake between Cl^- and NO_3^- is the primary culprit to decrease leaf NO_3^- concentration, and the lack of substrate (NO_3^-) restricts the NRA, since NR is a typical substrate-induced enzyme (Flores et al. 2000).

To compare both stresses, NR activity was significantly decreased by high Cl^- stress due to the antagonism with NO_3^-, but no influence was observed in osmotic stress (Fig. 6). However, the difference in NR activity did not affect biomass production, since both stresses always had identical biomass (Fig. 2). Therefore, this discrepancy is reasonably supposed to be a beneficial aspect of Cl^-, because a higher amount of Cl^- was accumulated in the developed leaf blade under high Cl^-, nearly 1.7 times as high as that of osmotic stress (Fig. 3a). Geilfus (2018a) reported that Cl^- could promote cell growth by regulating osmotic potential and turgor pressure.

In order to overcome this Cl^-/NO_3^- uptake competition under saline conditions, genetic modifications have been conducted in crops, *e.g.*, the overexpression of chloride channels (GmCLC1, GsCLC-c2) in the hairy roots of soybean which has been found to enhance salt tolerance. The over-expressed transport protein is highly correlated with the significant decrease of Cl^- concentration in shoots and then indirectly maintains a high NO_3^- accumulation in plant tissues. As a result, the ratio of Cl^-/NO_3^- remains constant in the stems and leaves of soybean (Wei et al. 2016, 2019). Nevertheless, whether this sole remedy is really sufficient to guarantee the metabolic function of NR in leaves under saline conditions remains unclear, because a preliminary question that if mass flow directly influence NO_3^- xylem translocation under osmotic stress should be firstly answered. If this effect depends on osmotic intensity or crop species, then the genetically modified strategy would not be working efficiently.

Conclusions

Maize is more sensitive to osmotic stress rather than Cl^- ion toxicity. Cl^- has beneficial effects on mitigating the osmotic stress via osmotic adjustment. Osmotic stress did not interfere NRA, since leaf NO_3^- concentration was not reduced. A link between Cl^- stress and NRA has been established in Cl^--stressed plants: the decreased NRA might be primarily caused by the antagonistic uptake of Cl^- and NO_3^-, finally resulting in the scarcity of metabolically available NO_3^- in leaf tissues.

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respectively. We also would like to show many thanks to the contribution of Mrs. Lilit Manukyan in the final language check.

Author contributions C-MG and CZ designed the concept and the experiments. XZ and LE conducted the experiments in the greenhouse and laboratory. BF supervised the cultivation of plants and took part in the data interpretation. XZ was responsible for data analysis, figures plotting, results discussion and manuscript drafting.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Supplementary figures

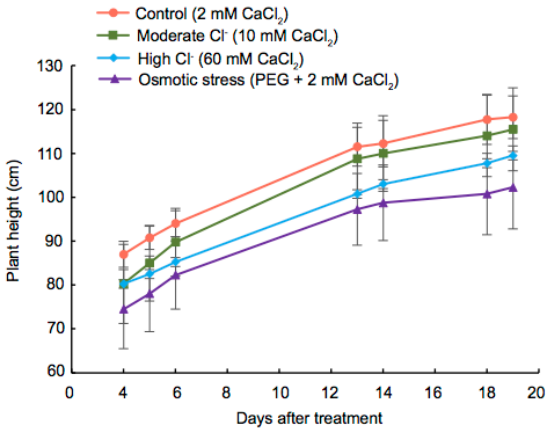


Fig.S1 Plant heights under different time-points after treatment
Plant heights were measured as the distance from the highest point to the soil surface at 4, 5, 6, 13, 14, 18 and 19 days after treatments. Values are expressed as mean value ± standard deviation (n=8).

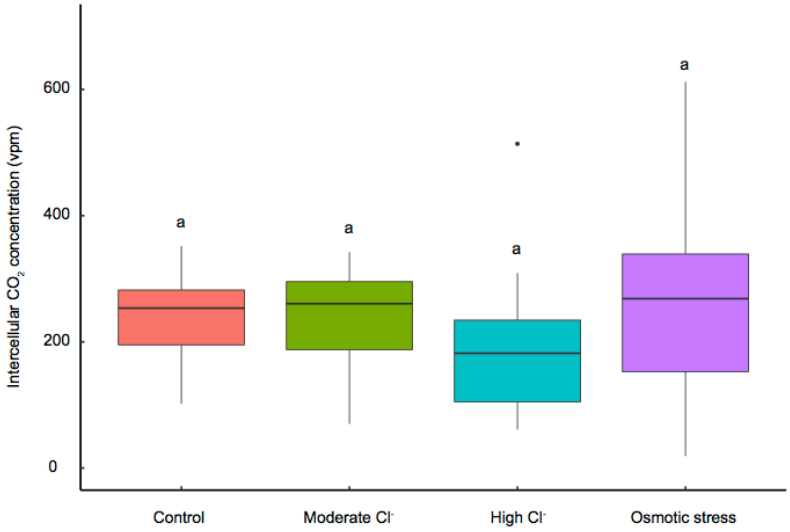


Fig.S2 Intercellular CO_2 concentration in the fully developed leaf blade
Average values of the 6th, 13th, 18th, and 19th days after the treatment are regarded as raw data for whisker-box plotting. Small letters represent significant differences ($P<0.05$, LSD test) in intercellular CO_2 concentration under different treatments (n=4).

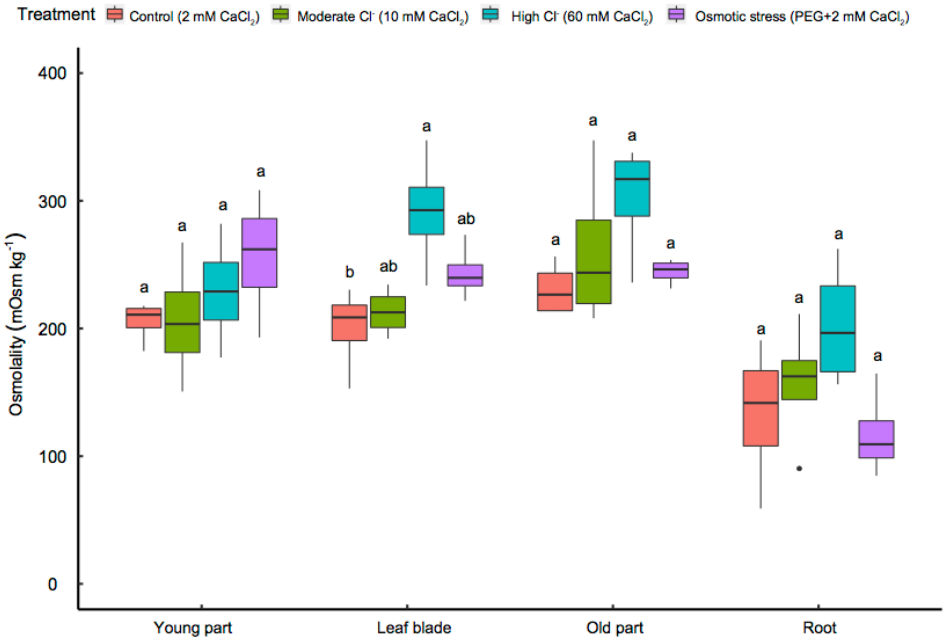


Fig.S3 Osmolality of various plant tissues in maize

The leaf sap was collected by squeezing the fully developed leaf blade. Sap was stored at -20° C. The osmolality of the extracts was measured with the vapor pressure osmometer VAPRO 5600 (Wescor, Inc, Logan, Utah, USA). The supernatant (10 µL) of leaf sap was pipetted onto a salt-free paper disc (SAMPLE DISCS SS-033, Wescor, Inc, Logan, Utah, USA). The osmometer was calibrated with 100 mmol kg^{-1}, 290 mmol kg^{-1} and 1000 mmol kg^{-1} OPTIMOLE osmolality standards (NaCl solutions) (Wescor, Inc, Logan, Utah, USA) before each measurement. The osmolality was described as the amount of osmotically active particles per mass of tissue material with the unit mmol osmolyte kg^{-1} fresh mass. Three technical replicates were conducted for each material. Small letters represent significant differences ($P<0.05$, LSD test) in one tissue group under the different treatments (n=4).

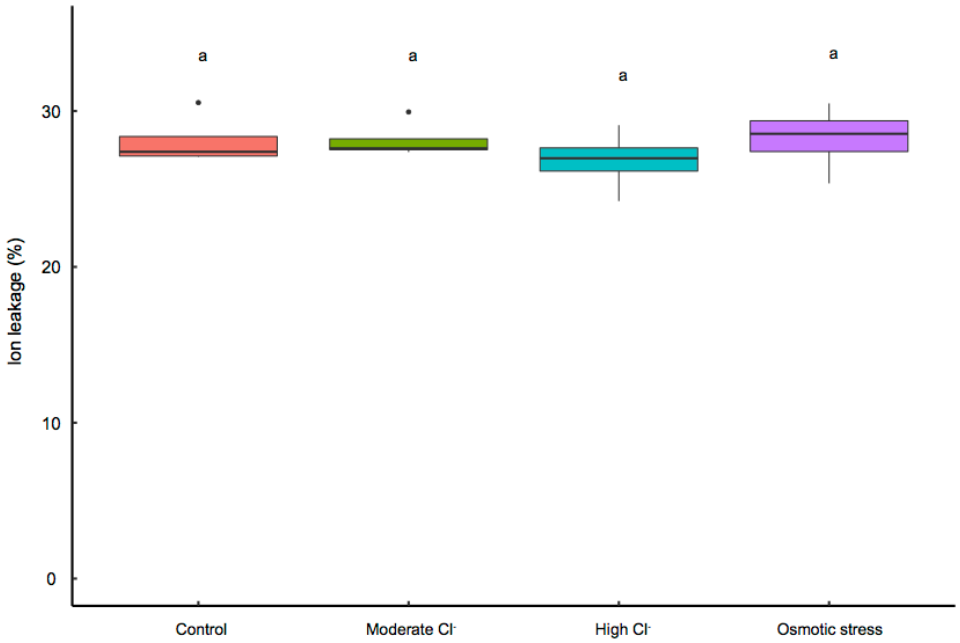


Fig.S4 Ion leakage of the fully developed leaf blade
Ion leakage was measured by a conductometer (WTW LF90 and a WTW KLE1 cell, Weilheim, Germany). When harvesting the plant material, six 1 cm diameter discs were immediately collected from the fully developed leaf blade and washed for four times with ddH_2O. Then, they were put into a 50 mL falcon tube containing 20 mL ddH_2O. The conductivity was firstly measured after 4 hours of shaking. The leaf discs were stored overnight at −20°C, and then the total conductivity was recorded after thawing. Ion leakage was expressed as the ratio of the conductivity (4 hours) and the total conductivity. Small letters represent significant differences ($P<0.05$, LSD test) in ion leakage under the different treatments (n=4).

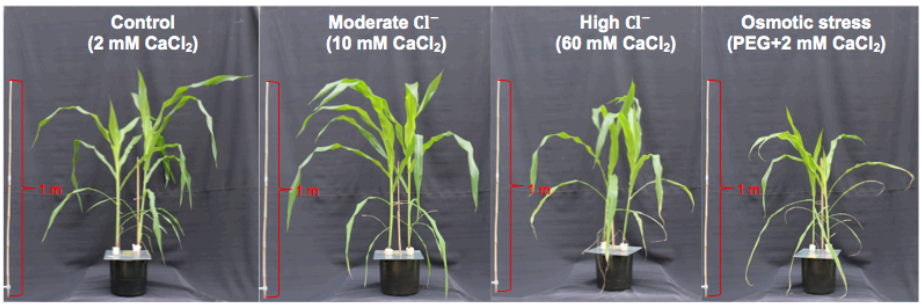
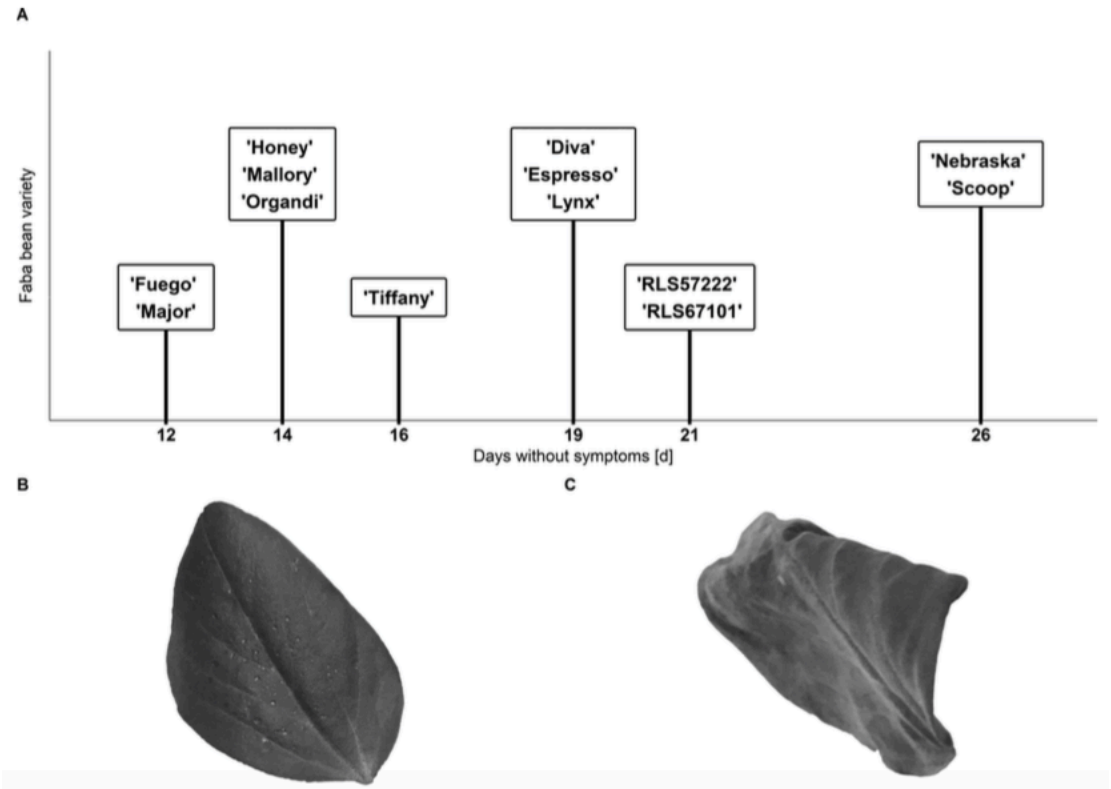


Fig.S5 Plant phenotypes of maize under the various Cl^- applications or osmotic treatment

7. Chapter IV

Shoot chloride translocation as a determinant for NaCl tolerance in *Vicia faba* L.



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Shoot chloride translocation as a determinant for NaCl tolerance in *Vicia faba* L.

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ABSTRACT

Faba bean (*Vicia faba* L.) is sensitive to salinity. While toxic effects of sodium (Na^+) are well studied, toxicity aspects of chloride (Cl^-) and the underlying tolerance mechanisms to Cl^- are not well understood. For this reason, shoot Cl^- translocation and its effect as potential determinant for tolerance was tested. Diverse *V. faba* varieties were grown hydroponically and stressed with 100 mM NaCl until necrotic leaf spots appeared. At this point, biomass formation, oxidative damage of membranes as well as Na^+, Cl^- and potassium concentrations were measured. The *V. faba* varieties contrasted in the length of the period they could withstand the NaCl stress treatment. More tolerant varieties survived longer without evolving necrosis and were less affected by inhibitory effects on photosynthesis. The concentration of Cl^- at the time point of developing leaf necrosis was in the same range irrespective of the variety, while that of Na^+ varied. This indicates that Cl^- concentrations, and not Na^+ concentrations are critical for the formation of salt necrosis in faba bean. Tolerant varieties profited from lower Cl^- translocation to leaves. Therefore, photosynthesis was less affected in those varieties with lower Cl^-. This mechanism is a new trait of interest for salt tolerance in *V. faba*.

1. Introduction

Faba bean (*Vicia faba* L.) contributes to nitrogen input to soil and is often used in organic agriculture (Köpke and Nemecek, 2010; Turpin et al., 2002). Faba bean is an important legume crop that is used as feed and food because of its high dietary protein content (Crépon et al., 2010). Legumes and particularly faba bean are sensitive to high salt loadings in soil resulting in limitations in yield and biomass (Li et al., 2017; Tavakkoli et al., 2010). Faba bean is grown in the Middle East, the Mediterranean region, China and Ethiopia. Some of these regions may face a problem with high salt loadings (Jensen et al., 2010), with sodium chloride representing the most soluble and prevalent salt (Butcher et al., 2016; Munns and Tester, 2008). The initial phase of salt stress is usually dominated by osmotic stress resulting from high solute concentrations and low soil water potential and is therefore categorized as 'osmotic-phase' (Munns and Tester, 2008). In response to the osmotic imbalance, faba bean undergoes fast physiological adaptations within the first hour (Geilfus et al., 2015a). In addition to rapid stomatal closure, metabolites associated with the formation and scavenging of reactive oxygen species accumulate, while a reduction in glutamine synthetase activity indicates disturbances in nitrogen assimilation, even in the early phase of salt stress. Additionally, a proline analogue (*trans*-4-hydroxy-L-proline) known to inhibit cell elongation is increasingly synthesized after NaCl-stress initiation (Geilfus et al., 2015b). In order to alleviate the effect of reduced cell expansion and lack of water, plants osmotically adapt by ion uptake (Amede et al., 2003; Farooq et al., 2015) and the synthesis of compatible solutes (Hasegawa et al., 2000; Kabbadj et al., 2017). With a continuous exposure to salinity, the accumulated salt ions lead to the disruption of ion homeostasis and cause symptoms of ion toxicity (Munns and Tester, 2008). Moreover, a clear separation into osmotic and ionic stress phases is problematic, because the transition between the phases is fluent and phases often overlap. In faba bean, high shoot Na^+ concentrations interfere with K^+ and calcium nutrition, while the accumulation of Cl^- is associated with a decline of photosynthetic capacity (Tavakkoli et al., 2010). The restriction of salt ion fluxes is mandatory both for preventing intra- and intercellular Na^+ and Cl^- concentrations from rising to toxic concentrations and for ensuring K^+ homeostasis. The latter is essential for the functioning of protein biosynthesis and the various cytosolic enzymes that might be impaired by competition of Na^+ and K^+ (Flowers

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et al., 2015; Tester and Davenport, 2003). One physiological strategy contributing to NaCl tolerance is salt ion exclusion at the root level and a restriction of ion transfer into the xylem to prevent them from being translocated acropetally towards the photosynthetically active leaves ('ion exclusion'). Another strategy is characterized by subcellular ion compartmentation into vacuoles or photosynthetically non-active cells to avoid the accumulation of harmful ions within the cytoplasm ('tissue tolerance') (Munns and Tester, 2008; Roy et al., 2014).

In faba bean, evidence has been presented illustrating variety-specific differences in the mechanisms regulating NaCl-based ion fluxes. In this study, a more tolerant genotype has been found to be capable of maintaining lower shoot Na^+ and Cl^- concentrations (Tavakkoli et al., 2010). However, information about intra-crop variance in terms of tissue tolerance is limited. When the internal Na^+ and Cl^- concentrations exceed the capacity of tissue tolerance, faba bean develops necrotic spots, starting on mature leaves. This is associated with a loss of photosynthetically active tissue that further compromises plant growth. This can occur after two weeks of growth under saline conditions in hydroponics, highlighting the sensitivity of the crop to salt (Slabu et al., 2009). In order to maintain growth under conditions of soil salinity, salt sensitive crops need to be improved with regard to their ability to withstand the negative soil properties that we expect to increase in the near future (Butcher et al., 2016; Wang et al., 2003).

The motivation behind this study was to evaluate on the basis of Cl^- and Na^+ accumulation pattern how key processes such as (*i*) ion retention and (*ii*) tissue tolerance contribute to increased performance under salinity. Another aim was to investigate the plasticity of the tolerance mechanisms of *V. faba* and to evaluate to which extent sensitive and tolerant varieties differ in their physiological stress response.

2. Materials and methods

2.1. Cultivation of plant material

For to screen a broad physiological range to salt tolerance thirteen diverse varieties of *Vicia faba* L. were selected for the study: 'Diva', 'Honey', 'Nebraska', 'Organdi' (Agri-Obtentions, Guyancourt, France), 'Espresso', 'Fuego', 'Lynx', 'Mallory', 'RLS57222', 'RLS67101', 'Scoop', 'Tiffany' (Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Hohenlieth, Germany) and 'Major' (Limagrain, Edemissen, Germany). This selection represents a broad genetic range of varieties of available German faba bean varieties from different breeding companies (population varieties). None of them was bred for salt tolerance. With the use of broadest genetic origin of these varieties we tend to evaluate the plasticity of stress reaction of faba bean under saline conditions. Plants were grown under hydroponic culture conditions in a walk-in climate chamber (14/10 h day/night; 22/18 °C; approx. 80–90% humidity, 500 µmol photons m^2 s^{-1} at shoot level). Seeds were imbibed in aerated $CaSO_4$ (0.5 mM) solution for 1 d at room temperature and were subsequently placed in moistened quartz sand. After 10 d of germination, seedlings were transferred into plastic containers containing 1/4-strength aerated nutrient solution. The nutrient concentration was increased stepwise to prevent osmotic shock. The nutrient concentration was increased to 1/2-strength after 2 d, 3/4-strength after 3 d and to full-strength after 4 d. Full-strength nutrient solution had the following composition: 0.1 mM KH_2PO_4, 1.0 mM K_2SO_4, 2.0 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.00464% (w/v) Sequestren (Ciba Geigy, Basel, Switzerland), 100 µM NaCl, 10 µM H_3BO_3, 2.0 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 0.1 µM $CoCl_2$, 0.05 µM $(NH_4)_6Mo_7O_{24}$. After 4 d of growth under the full-strength nutrient concentration, NaCl treatment was introduced to the 16-day-old plants. Starting from 1/3-strength, the NaCl concentration was incrementally increased to 2/3-strength after 2 d and to full-strength after 4 d (100 mM NaCl). Our previous experiments confirmed this application as moderate salt treatment for *V. faba* resulting in a moderate stress answer with moderate growth reduction and at the most the development of symptoms such as black spots only at sensitive varieties (Richter et al., 2015; Slabu et al., 2009). The solution was changed every third day to avoid nutrient depletion. For each variety, five biological replicate plants were cultivated under control and salt stress conditions.

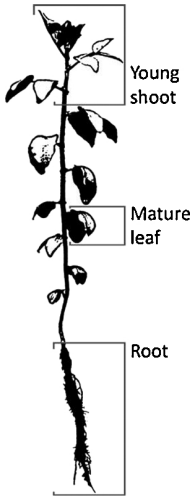

Fig. 1. Illustration of the sampled plant regions for the determination of biomass and ion analysis. Young shoot represents the plant region that started to develop under fullstrength NaCl treatment (100 mM). Mature leaf represents the 4th leaf at which leaf physiological measurements were conducted. The 4th leaf emerged under control conditions but developed during both the stress adaption phase and under conditions of 100 mM NaCl stress.

2.2. Transpiration, SPAD, biomass and electrolyte leakage

Assimilation (*A*) and transpiration (*E*) were measured by using a LC*i-SD* ultra compact photosynthesis system (ADC Bioscientific, U.K.) at the fully developed 4th leaf in the walk-in climate chamber (Fig. 1). The first measurement was conducted 3 d after the 100 mM NaCl stress was applied. Measurements were conducted in a randomized order and repeated every 2 to 3 d at the same time of a day (3 h after lights on). Chlorophyll content was estimated by SPAD values that were measured similarly to *A* and *E* by using a Minolta SPAD-502 Chlorophyll meter (Minolta Camera Co., Ltd., Osaka, Japan). Plants were harvested 1 d after necrotic salt lesions had occurred on the fully developed 4th leaves and when four out of five of the NaCl stressed biological replicates showed these symptoms. These symptoms were either (i) leaf necrotic spots (Fig. 2B) or (ii) visible loss of turgidity (Fig. 2C). The dry weights (DW) of total shoot, young shoot, 4th leaf and root were determined after drying to constant weight at 55 °C (Fig. 1). Electrolyte leakage (EL) was measured as described in Wedeking et al. (2017) by using four leaf discs (diameter of 0.8 mm) that had been cut from 5th leaf.

2.3. Potassium, sodium and chloride analysis

For ion extraction, a 50 mg sample of oven-dried plant material was solubilized in 8 mL 69% HNO_3 (v/v) and 4 mL H_2O_2 by microwave digestion at 190 °C for 25 min (MARS 5; CEM Cooperation, Matthews, NC, USA). To verify the extraction procedure, standard and blank samples were also digested. The digestates were filtered and analysed for K^+ and Na^+ concentrations by using an atomic absorbance spectrometer (3300 series; Thermo Fisher Scientific, Dreieich, Germany), whereas the Cl^- concentration was measured by using the ferricyanide method according to the protocol described by Munns et al. (2010).

2.4. Data analysis

Data were analysed by using *R* (R Development Core Team, 2017)

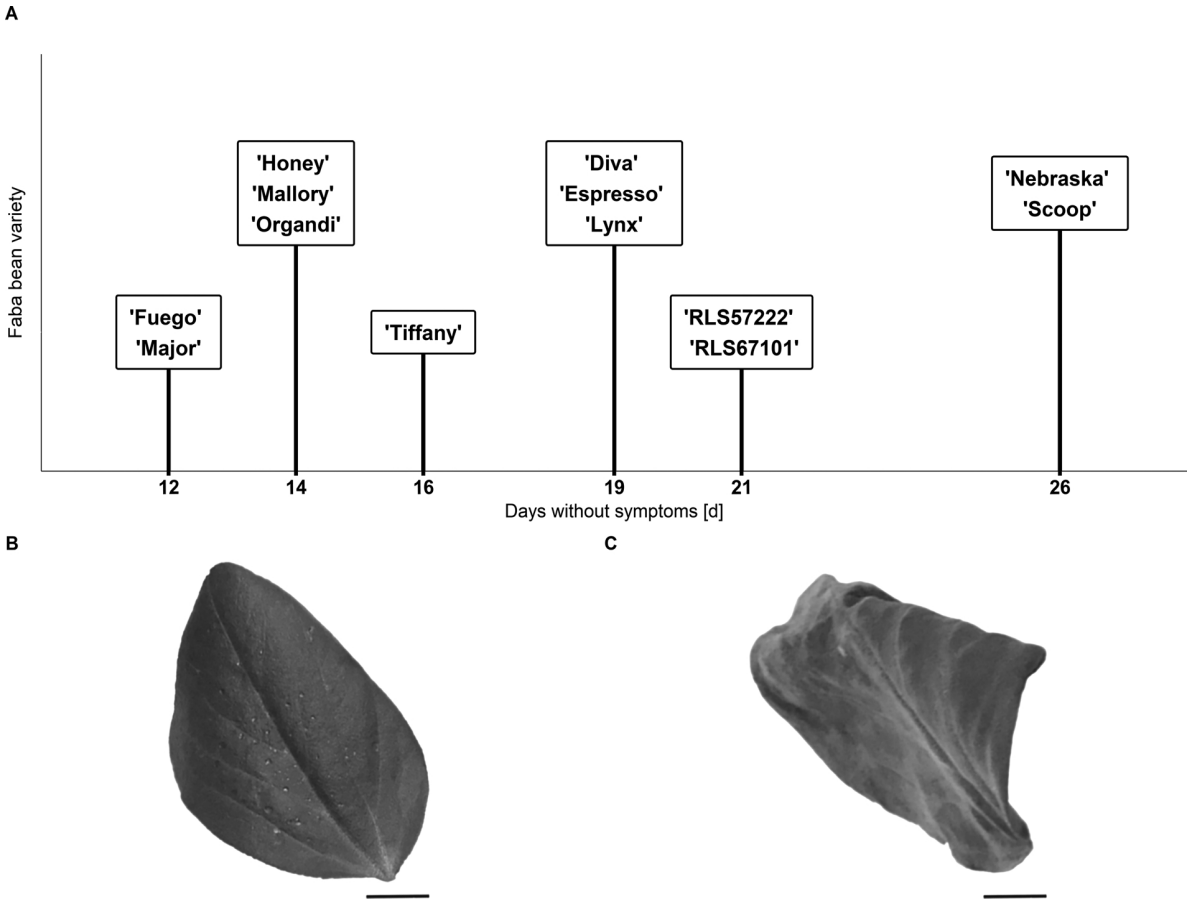

Fig. 2. Plasticity of faba bean varieties at 100 mM salt stress. A) Duration in days starting from full-strength salt application to development of leaf necrosis or loss of turgidity in at least four out of five salt-treated plants of a respective variety. Time point indicates harvest time; B) Faba bean leaf with necrotic spots. Image taken from a 4th leaf of 'Fuego' after 11 days of NaCl treatment; C) Faba bean leaf with loss of turgidity, from 3th leaf of 'Major' after 11 d of NaCl treatment. Scale bars represent 1 cm.

and the lme4-package to perform linear mixed effects analysis (Bates et al., 2015). As fixed effects, variety and treatment with an interaction term were entered into the model, whereas the positioning of the individual plants within the plastic containers of the hydroponics system was entered as a random effect. Residuals of statistical models were inspected visually and by using the DurbinWatson test from the car-package (Fox and Weisberg, 2011). Data were analysed on the basis of $p \leq 0.05$ by using the Tukey test algorithm and information of pairwise comparison and compact letter display was extracted by use of the multcompView package (Graves et al., 2015). Prior to clustering by using the stats package (R Development Core Team, 2017), optimal cluster number was estimated by using the fpc package (Hennig, 2015). Clustering was conducted according to the Hartigan and Wong algorithm with an estimated cluster number by optimum average silhouette width (R Development Core Team, 2017). Relative changes of dry weights were calculated relative to the non-stressed control group by subtracting log-transformed values. Changes of electrolyte leakage were calculated relative to the averaged non-stressed control group. Average ion accumulation was calculated as difference of salt stress concentrations and averaged non-stressed control group divided by the length of the exposure to NaCl stress. Data were visualized by using the ggplot2 package (Wickham, 2016), the ggbiplot package (Vu, 2011) and the corrplot package (Wei and Simko, 2017).

3. Results

3.1. Plasticity of faba bean varieties under salt stress

To identify the plasticity of stress physiological reactions and the range of diverse varieties in stress tolerance, faba bean varieties that contrast in their ability to withstand 100 mM NaCl stress were tested. Therefore, the experiment was conducted using a variable stress period, *i.e.* each variety grew as long as was needed to develop visible toxicity symptom. The plants of each variety were harvested when four out of five plants developed toxicity symptoms such as tiny leaf spot necrosis or severe loss of turgidity (Fig. 2B, C). By this, a comparison of the plasticity of the varieties at a similar physiological stress level was achieved. Salt-treated 'Major' plants suffered from severe loss of turgidity (Fig. 2C), whereas the other varieties developed tiny necrotic spots on leaves (Fig. 2B). The faba bean varieties differed in their stress response. Some of the varieties showed salt stress symptoms earlier, others later thus illustrating the contrast in their plasticity to withstand salt stress (Fig. 2A). In comparison of the 13 varieties 'Fuego' and 'Major' developed symptoms first (after 12 days of NaCl treatment). The other varieties developed leaf necrosis after 14, 16, 19, 21 and 26 days of NaCl treatment. The most tolerant varieties were 'Nebraska' and 'Scoop' which developed leaf necrosis after 26 days, this was 14 days later than the sensitive variety 'Fuego'.

3.2. Growth depression, membrane integrity, assimilation and transpiration

Of course, the absolute biomass formation increased with the

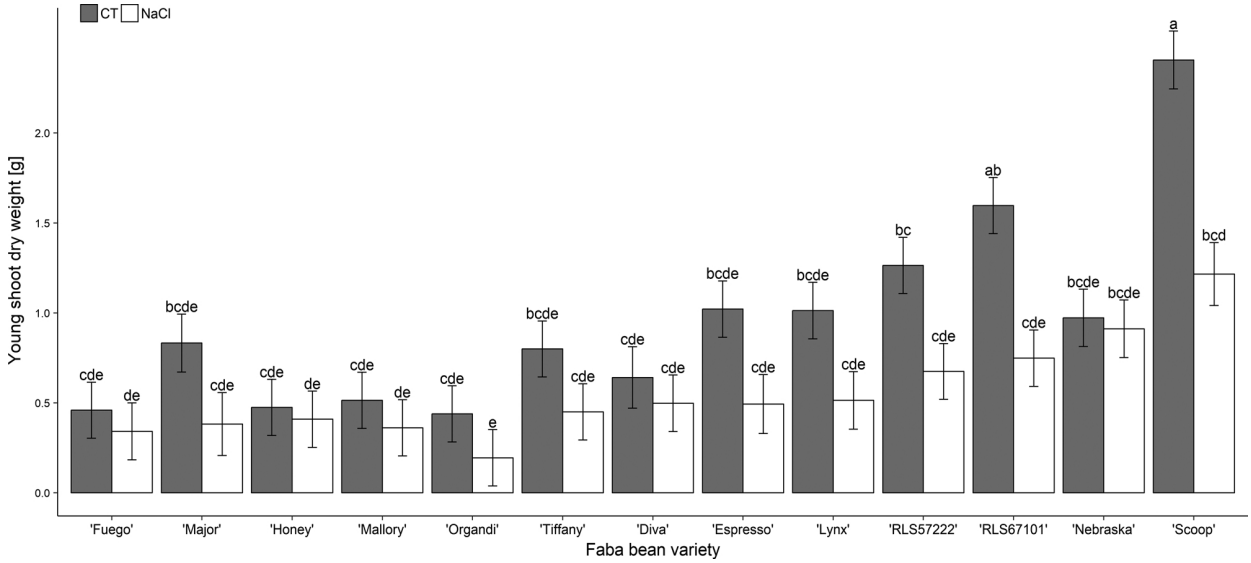

Fig. 3. Plasticity of faba bean varieties differing in young shoot biomass (at the individual day that the varieties displayed visible symptoms), dry weight of control (CT) and 100 mM NaCl. Plants were harvested when leaf necrosis or loss of turgidity occurred as indicated in Fig. 2. Adjusted means from linear mixed-effect model ± SE. Different letters indicate significant differences; $p \leq 0.05$; n = 5.

duration of the growth period under both control and NaCl conditions in all varieties showing that the treatment was moderate and not too harsh. The plasticity of the varieties was high because salt-induced growth depression ranged in average from 5 to 65% in young shoots that had developed under the influence of salt stress. However, growth depression was only significant for 'RLS67101' and 'Scoop' (Fig. 3). The most tolerant variety 'Nebraska' (Fig. 2A) did not show significant growth depression neither necrosis within 25 d of stress duration.

The status of the membrane integrity of mature leaf was assessed by measuring their electrolyte leakage. The electrolyte leakage was significantly increased by NaCl treatment in all varieties with the exception of the early salt-injury-developing varieties 'Fuego' and 'Honey', which had similar leakage rates under control and salt conditions (Fig. 4). With the exception of the two mentioned varieties, the relative increase of electrolyte leakage was at a similar level although the exposure time to NaCl varied (Fig. 2A).

Leaf transpiration rate (*E*) and CO_2 assimilation rate (*A*) were measured every 2nd or 3rd day, starting 3 d after full-strength NaCl application (Fig. 5). In comparison with control, salt treated plants had significantly reduced *E* and *A* with an average reduction of about 70% and 30%, respectively (Fig. 5). At the early stress phase (3 d since full-strength NaCl application), salt treated plants showed reduced *E* of about 1 mol H_2O m^{-2} s^{-1} that fluctuated only a little in the following days of the stress period. Conversely, *A* was about 5.5 µmol CO_2 m^{-2} s^{-1} and decreased significantly for most varieties in the subsequent period of 5 to 10 d of NaCl stress to rates of about 2 µmol CO_2 m^{-2} s^{-1} (Fig. 5). Two days after *A* decreased to this level, the varieties such as 'Fuego', 'Honey' and 'Mallory' already had developed necrotic spots. The *E* of 'RLS67101', 'Nebraska' and 'Scoop' was slightly higher in comparison with the other varieties. Furthermore, *A* decreased only to approx. 2.5 µmol CO_2 m^{-2} s^{-1} and did slightly recover to 3.5 µmol CO_2 m^{-2} s^{-1} in the period from 14 to 17 d of salt stress. The latter trend was also visible at 'Diva' and 'Espresso'.

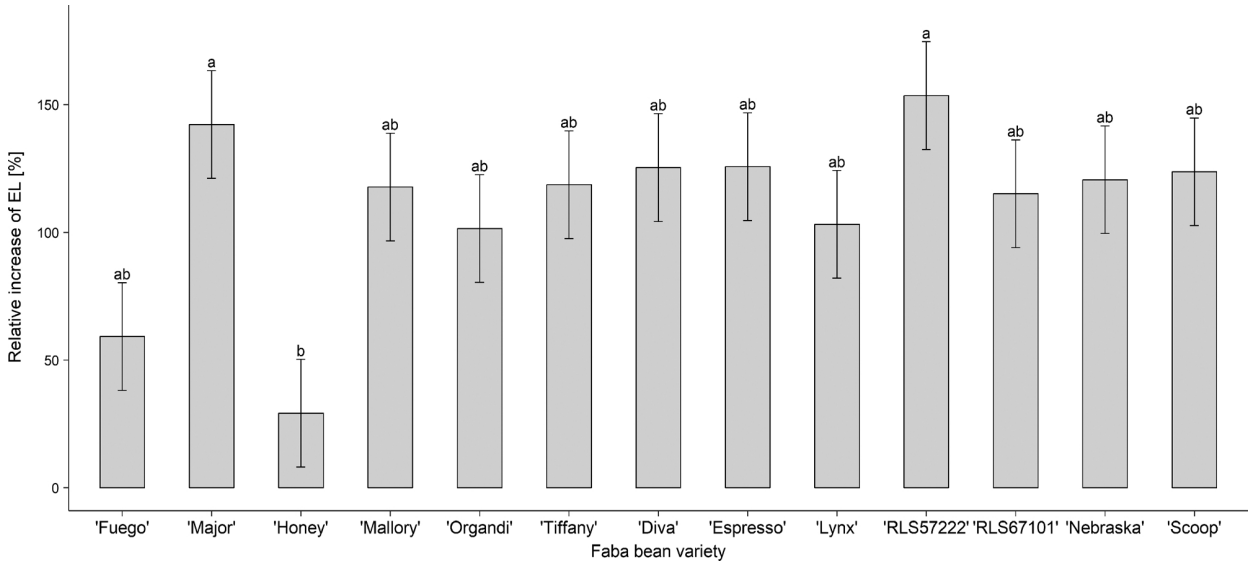

Fig. 4. Membrane integrity of faba bean varieties. EL, relative increase of electrolyte leakage after NaCl treatment in comparison to unstressed controls. Samples were taken when first symptoms occurred as indicated in Fig. 2. Means ± SE. Different letters indicate significant differences; $p \leq 0.05$; n = 5.

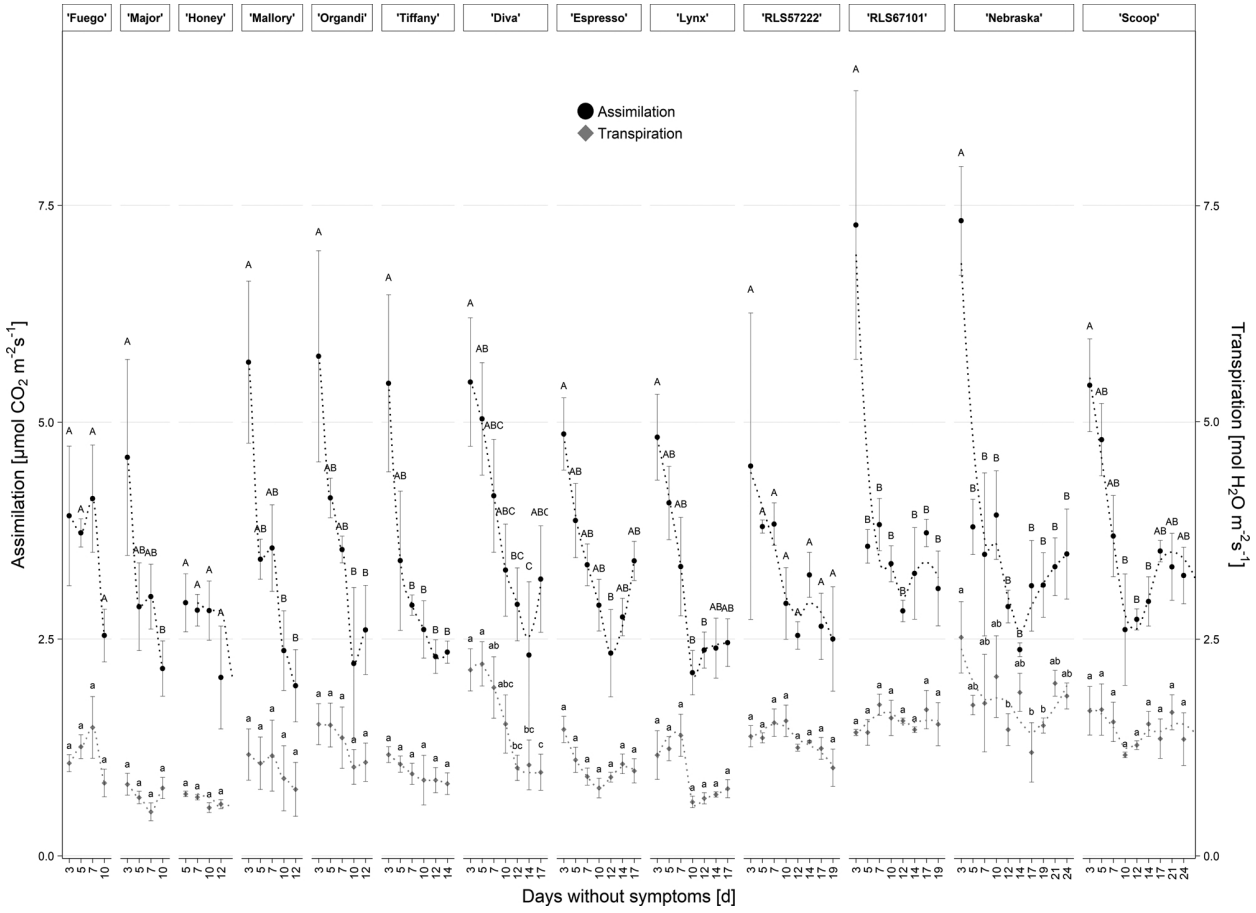

Fig. 5. Assimilation (A) and transpiration rate (E) of faba bean varieties grown at 100 mM NaCl. Measurements were conducted at mature leaves (4th) starting from 3 days after full-strength salt stress until the respective variety developed symptoms. Means ± SE with dotted trend line (local polynomial regression fit). Different letters indicate significant intra-variety differences of means; $p \leq 0.05$; $n = 3$.

3.3. Ion pattern

The concentrations of potassium [K^+], sodium [Na^+] and chloride [Cl^-] were analysed to determine plasticity of tissue specific ion patterns in these varieties. Sodium in control plants was hardly detectable, whereas the average [Cl^-] ranged from 150 to 200 µmol g DW^{-1} in young shoots and from 100 to 420 µmol g DW^{-1} in mature leaves (Figs. 6A, 7 A). The [K^+] was similar for most varieties, with concentrations ranging from 1050 µmol g DW^{-1} in young shoots to 1150 µmol g DW^{-1} in mature leaves of control plants (Figs. 6A; 7 A). The addition of 100 mM NaCl to the nutrient solution resulted in an accumulation of salt ions but the increase of Na^+ was about two-fold higher than that of Cl^- in both young shoots and mature leaves (Figs. 6A; 7 A). In mature leaves, [Na^+] and [Cl^-] were higher than in young shoots. In contrast, the reduction of [K^+] was more pronounced in mature leaves that had accumulated 84% more Na^+ than young shoots (Figs. 6A; 7 A). In comparison with the later symptom-developing varieties 'RLS67101', 'Nebraska' and 'Scoop', the early symptom-developing variety 'Fuego' accumulated less Na^+ in young shoot and mature leaf.

The pattern of Na^+ and K^+ concentrations in young shoots differed in relation to the duration that the plants grew under stress. The [Na^+] increased with the duration of NaCl stress ('days without symptoms') whereas that of [K^+] decreased (Suppl. Fig. 1). The varieties 'Nebraska' and 'Scoop', which developed symptoms later, showed higher [Na^+] in young shoots and mature leaves. These both varieties had also lower [K^+] compared with the more sensitive variety 'Fuego' which developed necrotic spots earlier (Fig. 6A). In contrast, the [Cl^-] differed much less at the time point when leaf necrotic spots appeared. This pattern was visualized by a density plot: in young shoots and mature leaves the densities of [Cl^-] were about double in comparison to [Na^+] (Figs. 6B; 7 B). However, in mature leaves the density of [Cl^-] was twice as high compared with young shoots (Fig. 7B). Moreover, the pattern for Cl^- shows narrow density peaks in comparison with Na^+ with broad peaks. In particular, [Cl^-] in young shoots was similar, irrespective of the variety and the various exposure time to NaCl (Fig. 6B). The common pattern was that plants, regardless of their variety, formed salt-stress symptoms at a specific Cl^- tissue concentration, whereas such a pattern was not observed for Na^+ (Figs. 6B; 7 B).

To find a tissue ion pattern for the diverse varieties a hierarchical clustering according to their Na^+ and Cl^- concentrations was done (Fig. 8A, B). In both leaf fractions (young and mature), varieties with lower ion concentrations clustered into one group (dark triangle) and other varieties with 1.5 to 2-fold higher tissue ion concentration clustered together (light triangle). Varieties such as 'RLS67101', 'Nebraska', and 'Scoop' developing symptoms later clustered into the group with higher tissue ion concentrations. However, [Cl^-] in young shoots was similar in all varieties except for 'Major' (Fig. 8A), which did not develop necrotic spots but showed loss of turgidity (Fig. 2C). Ion tissue concentrations in mature leaves were in general higher compared to young tissue and a diversification of the concentration pattern of all varieties was observable.

The average ion accumulation per day of NaCl stress exposure (stress dose) was calculated to relate the ion concentration at the time point of the appearance of symptoms to the length of the respective stress period. This value serves as an evaluation for the height of the daily ion-accumulation to show the potential differences between Na^+

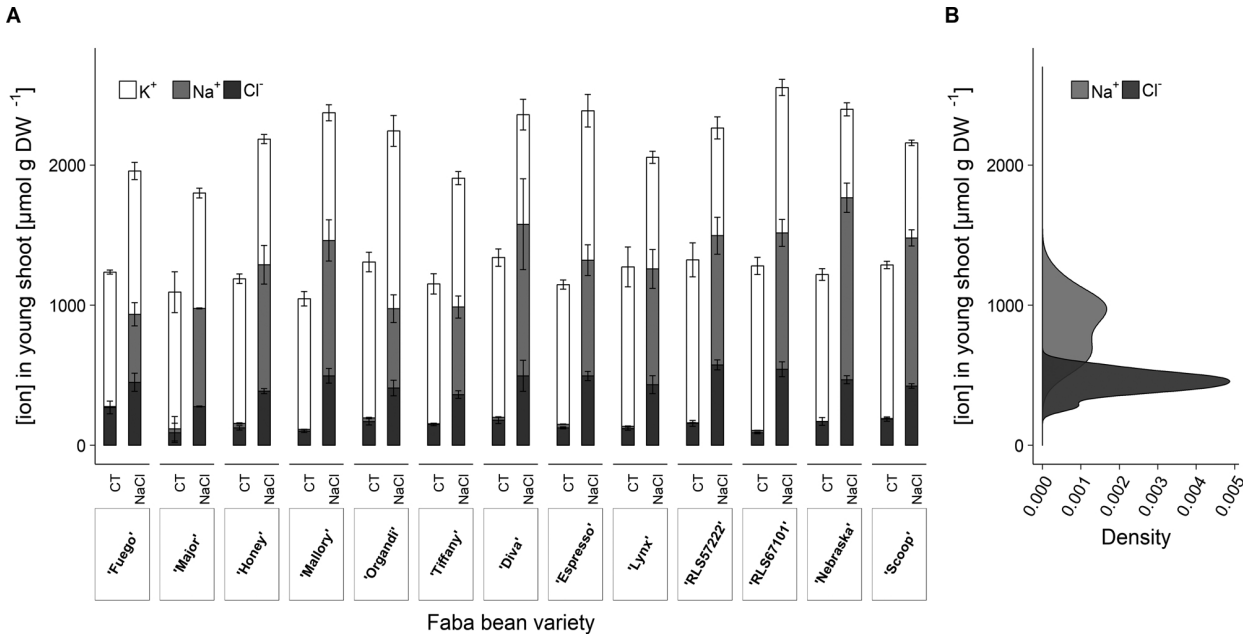

Fig. 6. Ion concentrations in young shoot of faba bean. A) Concentrations of sodium Na^+, chloride Cl^- and potassium K^+, non-stressed controls (CT), 100 mM NaCl (NaCl). B) density plot of Na^+ and Cl^- concentrations in young shoots of 13 averaged NaCl-treated faba bean varieties. Samples were taken when leaf necrosis or loss of turgidity occurred as indicated in Fig. 2. Adjusted means from linear mixed-effect model ± SE; n = 5.

and Cl^- intake of the diverse varieties. The average Na^+ accumulation in young shoot was similar in all varieties with values ranging from 40 to 60 µmol g DW^{-1} d^{-1} (Fig. 8C). Cl^- was accumulated up to 20 µmol g DW^{-1} d^{-1}. The variety 'Mallory' was significantly different to 'Scoop' that had lowest average accumulation of 10 µmol g DW^{-1} d^{-1}. In mature leaves, the average Na^+ accumulation was similar to that of young shoots for all varieties, ranging from 100 to 140 µmol g DW^{-1} d^{-1}, except of 'Honey' with 50 µmol g DW^{-1} d^{-1} (Fig. 8D). In most varieties, Cl^- accumulated up to 40 µmol g DW^{-1} d^{-1}, except for 'Mallory', 'Nebraska' and 'Scoop' which had lower values of about 25 µmol g DW^{-1} d^{-1}. The relative Na^+/K^+ ratios in young shoots and mature leaves increased with NaCl treatment (Fig. 8E, F). Highest increases in young shoot of 600-fold and 200-fold were found in those varieties ('Nebraska', 'Scoop', 'RLS57222') that developed symptoms later than others (Fig. 8E). In mature leaves, the relative Na^+/K^+ ratios of the earlier-symptom developing varieties ('Fuego', 'Major', 'Honey') increased by about 60 to 100-fold whereas the other varieties showed increases of 200-fold or higher (Fig. 8F).

Analysis of principle components (PCA) based on concentrations of Na^+, Cl^- and K^+ revealed that the more salt-sensitive 'Fuego', 'Honey' and 'Organdi' were separated due to higher [K^+] and lower [Na^+], whereas the contrary was found for the later symptom-developing

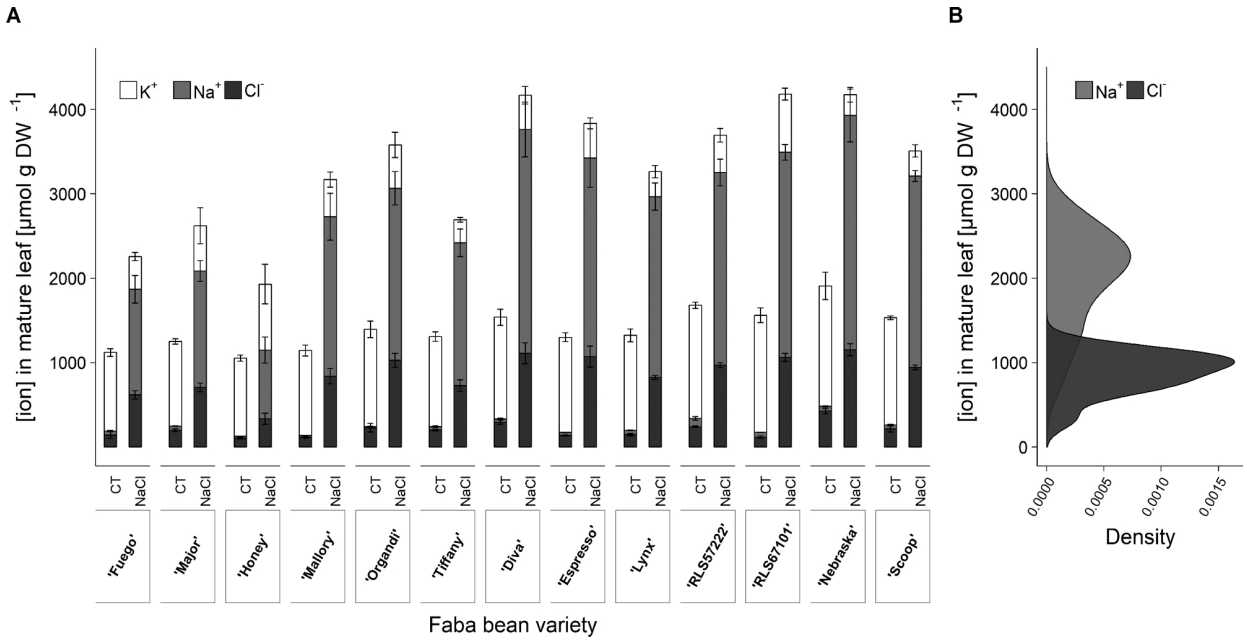

Fig. 7. Ion concentrations in mature leaf (4th) of faba bean. A) Concentrations of sodium Na^+, chloride Cl^- and potassium K^+, non-stressed controls (CT), 100 mM NaCl (NaCl). B) density plot of Na^+ and Cl^- concentrations in mature leaves of 13 averaged NaCl-treated faba bean varieties. Samples were taken when leaf necrosis or loss of turgidity occurred as indicated in Fig. 2. Adjusted means from linear mixed-effect model ± SE; n = 5.

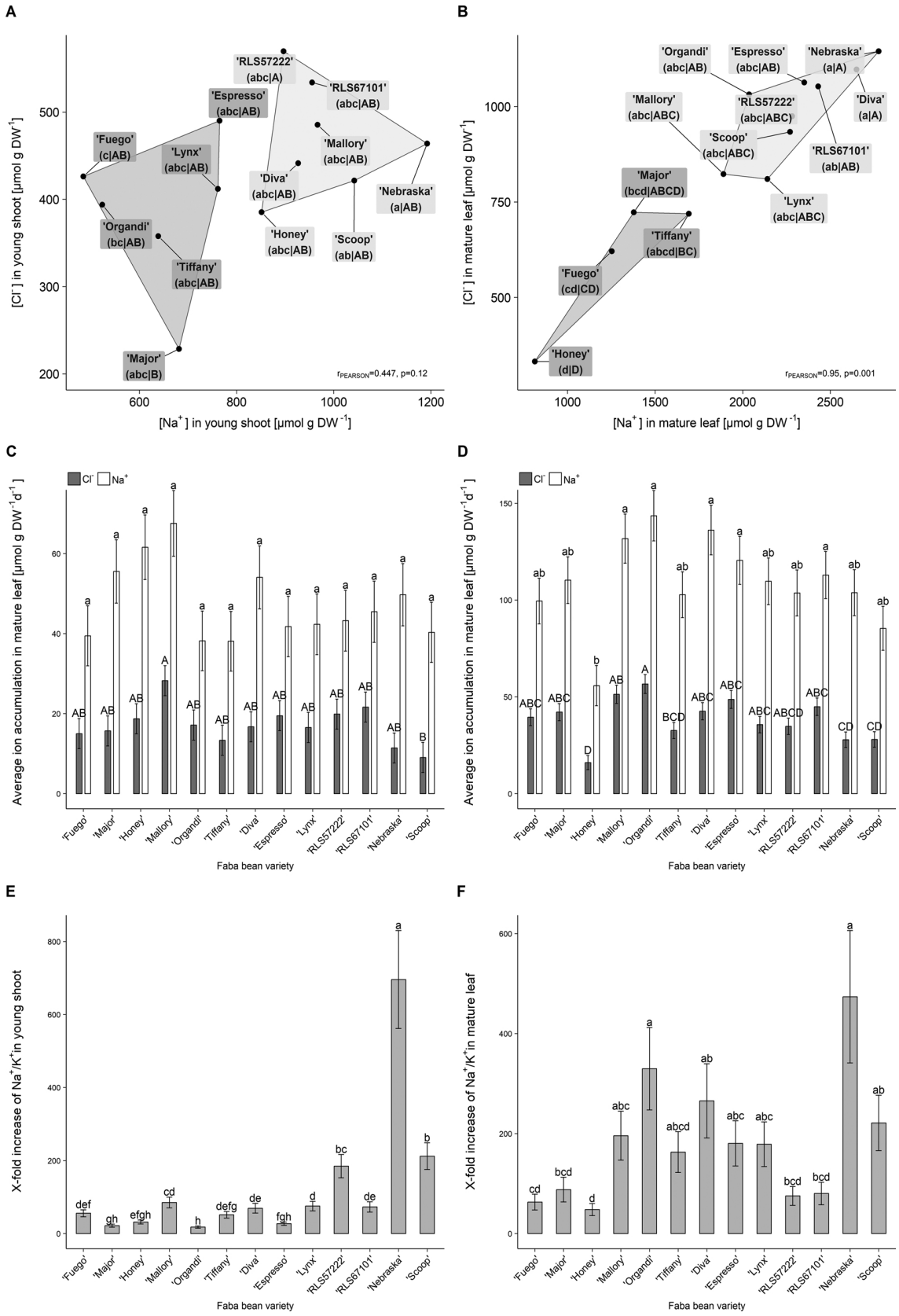

Fig. 8. Comparison of faba bean varieties by means of ion composition at one day after the development of symptoms (Fig. 2). A) Clustering of NaCl-treated faba bean varieties based on concentrations of sodium Na^+, chloride Cl^- and potassium K^+ in young shoot and B) mature leaf. C) Average Cl^- and Na^+ accumulation per day of NaCl application in young shoot and (D) mature leaf. E) Relative increase of Na^+/K^+ ratio in response to salt treatment in young shoot and (F) mature leaf. A–B) Adjusted means from linear mixed-effect model; C–F) Means ± SE. Different letters indicate significant differences: capital letters: Cl^-; lowercase letters: Na^+; $p \leq 0.05$; $n = 5$.

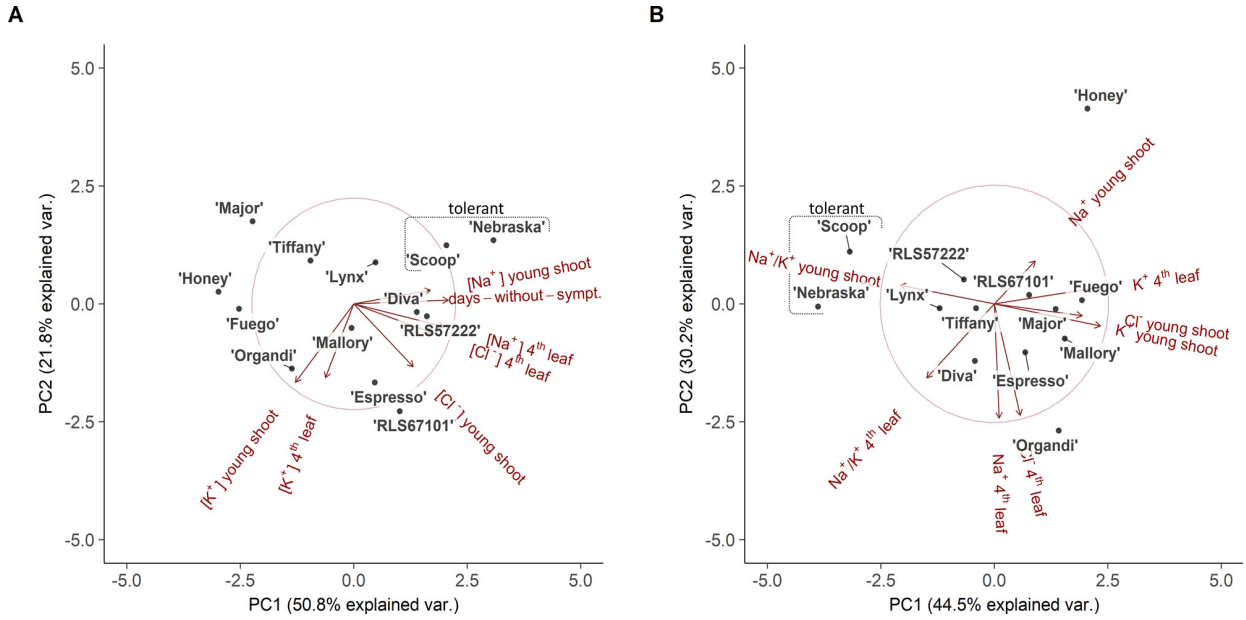

Fig. 9. Principal component analysis of NaCl-treated faba bean varieties A) based on concentrations of sodium Na^+, chloride Cl^- and potassium K^+ in young shoot and mature leaf and B) average Cl^- and Na^+ accumulation per day of NaCl application ('days without symptom') and relative increase of Na^+/K^+ ratio in response to salt treatment in young shoot and mature leaf.

varieties 'Nebraska' and 'Scoop' (Fig. 9A). The variety 'RLS67101', also belonging to the later symptom-developing varieties was characterized by higher $[K^+]$ combined with increased $[Cl^-]$ and $[Na^+]$ in mature leaves. Due to increased $[Na^+]$ and $[Cl^-]$ combined with low $[K^+]$, 'Nebraska' and 'Scoop' were at the right side (Fig. 9A). On the contrary, the variety 'Major' was separately arranged due to lowest $[Cl^-]$ in young shoot. PCA based on the calculated measures, average ion accumulation and increase in Na^+/K^+ ratio, illustrated that the later symptom developing varieties 'Nebraska' and 'Scoop' were separated due to low average shoot Cl^- and K^+ accumulation and highly increased shoot Na^+/K^+ ratio (Fig. 9B). Conversely, the early symptom developing 'Fuego' was at the right side due to higher average shoot Cl^- accumulation as well as higher K^+ accumulation in young shoot and mature leaf (Fig. 9B).

4. Discussion

4.1. Plasticity of NaCl tolerance in Vicia faba

In contrast to common experimental setups using fixed stress exposure time, our experiment was conducted with synchronized stress level to characterize the diverse faba bean varieties as salt sensitive or more tolerant and further evaluate on the basis of ion accumulation patterns how salt ion exclusion and retention mechanisms could have contributed to the observed differences in salt stress tolerance. With this approach it is possible to evaluate the plasticity of either sensitive or more tolerant genotypes differing in their physiological stress response.

Our experimental results show that faba bean varieties differ in their ability to withstand salinity. The plasticity of tolerance to salinity of *V. faba* seems to be broad and even without having the breeding goal of salt tolerance in these German varieties there is a high genetic and physiological variance within this trait. In response to prolonged salt treatment, all varieties except of 'Major' developed necrotic lesions on leaves. The necrotic spot symptom is attributed to the ionic part of salt stress which is an addition of an ionic imbalance together with an ion toxicity caused by high Na^+ concentrations and excess Cl^-. The plant either has to exclude excess ions or deal with these physiological inconveniences. This can be achieved by a so called 'tissue tolerance'. The differences in Na^+/K^+ ratio of the diverse varieties also showed the plasticity of *V. faba* in terms of dealing with ion homeostasis or excessive salt ion accumulation that may lead to cytotoxicity (necrosis) and consequently the loss of photosynthetically active tissue (Fig. 2B) (Geilfus, 2018; Slabu et al., 2009). In faba bean, this 'overcome of tissue tolerance' is a sudden process, as necrotic spots appeared mostly overnight and resulted in leaf senescence within about three days. The difference among sensitive and tolerant varieties was approximately double in NaCl dose that caused symptoms, therefore we consider the plasticity of *V. faba* also as relatively high in terms of salt tolerance mechanisms. In contrast, in the literature *V. faba* is considered as salt sensitive crop (Li et al., 2017; Maas and Hoffman, 1977; Slabu et al., 2009). We think it is of great matter which variety has been used to evaluate this. Of note, the variety 'Major' showed a loss of turgidity and wilting, which represent symptoms that are attributed to osmotic stress (Fig. 2C). In that physiological stage, ion accumulation had likely not yet exceeded the tissue tolerance capacity.

By choosing this experimental setup, salt tolerant plants were evaluated according to their variety-specific growing days without symptoms. On the basis of the temporal difference in development of symptoms referred to as days without symptoms, the variety 'Fuego' was identified as salt sensitive whereas 'Nebraska' and 'Scoop' were more tolerant (Fig. 2A). As biomass increased with growth time (Suppl. Fig. 1), the tolerant varieties tended towards formation of higher biomass of the young shoot fraction, that had developed under full-strength NaCl treatment (100 mM NaCl) (Fig. 3). Interestingly, the shoot growth of the tolerant variety 'Nebraska' appeared to be unaffected by salt treatment although the respective plants had faced the longest stress period before developing symptoms. However, this might be due to a comparatively low growth performance also under control conditions and should therefore not lead to the erroneously conclusion that the variety 'Nebraska' is preferable to that of 'Scoop' in terms of biomass production in saline environments (Fig. 3).

4.2. Oxidative stress, assimilation and transpiration

In order to evaluate physiological parameters such as membrane integrity under stress conditions, we compared variety-specific increases in electrolyte leakage. These are ascribed to stress conditions such as salinity, drought or pathogen attack (Demidchik et al., 2014;

Miller et al., 2010). This membrane damage results from oxidative processes or stress-related decrease of the lipid to protein ratio (Borochov-Neori and Borochov, 1991; Dionisio-Sese and Tobita, 1998). The maintenance of low electrolyte leakage under stress conditions has been associated with tissue tolerance (Arefian and Malekzadeh Shafaroudi, 2015; Bose et al., 2014; Lee and Zhu, 2010; Sudhakar et al., 2001). In our study, electrolyte leakage was measured at the time point when salt lesions appeared meaning that the variety-specific capacity of tissue tolerance was overcome. In this context, electrolyte leakage represents a measure for the intensity of oxidative stress that the plants had been exposed to. In response to salt treatment, all varieties except for 'Major' and 'Honey' had similar electrolyte leakage when salt injuries occurred (Fig. 4). This indicates that the level of oxidative stress was similar for most varieties at the time point when leaf necrosis appeared. However, the membrane integrity of 'Fuego' and 'Honey' was less hampered under conditions of salt stress, but the two varieties were able to endure this stress only for 12 and 14 days, respectively. Because those varieties that developed symptoms later under stress conditions had increased relative electrolyte leakage, we conclude that impaired membrane integrity is a consequence of increasing salt ion accumulation but does not necessarily lead to the formation of leaf necrosis.

Leaf transpiration (*E*) and assimilation rate (*A*) are non-destructive measurements representing a physiological indicator for salt tolerance that allows conclusions to be made with regard to the health and functional integrity of leaves (Munns et al., 2016). We performed these non-destructive physiological measurements at the 4th leaves, which later were analyzed for their K^+, Na^+, and Cl^- concentrations for better comparison (Fig. 7A). Faba bean growing under saline conditions responded by reducing *E* to avoid undesired water loss (Fig. 5) (Geilfus et al., 2015a; Keisham et al., 2018; Roy et al., 2014). Unlike to our expectations the more tolerant varieties maintained even higher *E* than sensitive ones, in contrast one would expect that a reduction in *E* is an essential attribute to save water (Fig. 5). Moreover, as stomata regulate access of CO_2 to photosynthetic active tissues, a decreased stomatal conductivity is assumed to compromise assimilation rate by restricting CO_2 diffusion into leaves (Lawson and Blatt, 2014). Although *E* and therefore CO_2 influx did not decrease or only slightly decrease since the continuing stress period, a significant decrease of *A* was seen from 3 to 5 days after full-strength salt stress application for some varieties. Especially the salt sensitive varieties 'Fuego', 'Major' and 'Honey' showed this trend of a continuously decreasing *A* until salt lesions had occurred. In contrast, the tolerant varieties maintained higher *E* and *A* during prolonged exposure to salt (Fig. 5). Besides the inhibitory effect of osmotic stress that results in photorespiration and decrease of *A*, an unbalanced chloroplastidial Cl^- homeostasis is expected to compromise photosynthesis. Under salinity, field bean accumulates Cl^- in chloroplasts that might reduce photosynthetic quantum yield by reduction in chlorophyll content (Slabu et al., 2009; Tavakkoli et al., 2010). In addition, the inhibition of CO_2 fixing enzymes, disturbed dark relaxation of chloroplasts, damage of PSII reaction centers due to photoinhibition as well as excessive production of ROS in chloroplasts were associated with excess chloroplastidial Cl^- (Geilfus, 2018). Hence, a decreasing *A*, which is not directly limited by reduced stomatal conductivity, might be explained by excess Cl^- that had been translocated into the shoot and ultimately accumulated in chloroplasts. This explanation is consistent with the differences in variety-specific Cl^- translocation to the young shoot and mature leaves (Fig. 7C, D), which potentially enabled the tolerant varieties to protect their sites of primary photosynthesis more efficiently from excess Cl^- intake and thus may help preventing chlorophyll degradation and the resulting decrease of *A* (Fig. 5; Suppl. Table 1).

4.3. Tolerant *V. faba* varieties sequester Na^+ ions

All varieties accumulated Na^+ and Cl^- ions but to a different extent (Figs. 6A, 7 A). The Na^+ concentration increased with the length of NaCl-stress exposure. Consequently, longer growing varieties had higher Na^+/K^+ ratios compared with NaCl-sensitive varieties such as 'Fuego' (Fig. 7E, F). In particular, the most tolerant varieties 'Nebraska' and 'Scoop' showed highest and second highest relative Na^+/K^+ ratios in both young shoots and mature leaves (Figs. 8E, F; 9 B). Sodium is often referred to as the most toxic ion in plants at saline conditions, because high cytosolic concentrations disturb K^+-homeostasis and therefore interfere with enzyme function and the regulation of stomatal aperture under saline conditions (Deinlein et al., 2014; Flowers et al., 2015; Hasegawa, 2013; Munns et al., 2016). However, the ion pattern at the time point of a similar stress level revealed that the maintenance of a low Na^+/K^+ ratio seems to be a less important attribute for salt tolerance in faba bean. This finding is in line with previous work in which legumes were associated with Cl^- sensitivity (Geilfus, 2018; Li et al., 2017; Teakle and Tyerman, 2010). We found highly increased relative Na^+/K^+ ratio in leaves of such varieties that showed earlier leaf necrotic spots such as 'Mallory' and 'Organdi' (Fig. 7E, F) and in varieties that developed symptoms the latest, such as 'Scoop' and 'Nebraska'. This implies that the shoot Na^+ translocation of those varieties was less controlled, meaning that tolerance in terms of Na^+ exclusion and retention was less pronounced. Therefore, mechanisms enabling tolerance to Na^+ in faba bean occurred most probably as tissue tolerance, since the Na^+ accumulation appeared to predominantly depend on the length (dose) of the salt exposure. As a consequence, excessive Na^+ in the shoot needed to be effectively sequestered because K^+ homeostasis is essential for cell function (Munns et al., 2016; Zörb et al., 2014). The ability to maintain K^+ homeostasis in root and leaf tissues and thereby protecting balanced cytosolic Na^+/K^+ ratio under saline conditions represents an important trait contributing to salt tolerance (Hauser and Horie, 2010; Wu et al., 2018). Hence, those varieties showing highly increased Na^+/K^+ ratios must have been effectively compartmentalizing Na^+ away from the cytosol, *e.g. via* compartmentalization into the vacuole (Munns et al., 2016; Percey et al., 2016). This makes ions to a certain extent available as 'cheap' osmolytes (Blumwald, 2000; Keisham et al., 2018; Niu et al., 1995). The cheap usage of salt ions for osmotic adjustment represents a physiological adaption of naturally salt tolerant halophytes and preserves energy for growth, as the synthesis of organic solutes is energetically more expensive. Further, sequestering Na^+ into the vacuole is preferential over the removal into the apoplast, as it contributes to membrane potential *via* removing positive charge from the cytosol. Thereby, the relocation of K^+ from the vacuole into the cytosol and the retention of cytosolic K^+ is favored (Percey et al., 2016). Thus, the later symptom-developing field bean varieties may have been able to effectively sequester Na^+ ions into the vacuole enabling an increased K^+ retention and a better control of their water status (Fig. 5) (Flowers and Colmer, 2008; Mancarella et al., 2016; Pan et al., 2016; Wu et al., 2018).

4.4. Leaf necrosis appears at distinct Cl^- concentrations in all *V. faba* varieties

Many previous salt-stress experiments were rather focused on Na^+ than Cl^-. However, in few papers legumes have been reported to be sensitive to Cl^- (Li et al., 2017; Teakle and Tyerman, 2010). In addition to the role of Cl^- in the initiation of stomatal closure during the early NaCl-stress response (Geilfus and Mühling, 2013), some evidence has been found for Cl^- being the predominant toxic ion in faba bean, affecting photosynthesis and plant growth to a greater extent than Na^+ (Slabu et al., 2009; Tavakkoli et al., 2010). Besides indications on the adverse effects on *A* (Fig. 5), we have found that irrespective of the duration until symptoms occurred, all varieties, *e.g.* those growing 12 or 26 days, had comparable [Cl^-] in young shoots and mature leaves that were harvested when the plants had developed leaf necrosis (Figs. 6B; 7 B). Based on this observation and in line with previous work we conclude that the [Cl^-] in developing leaves might be the critical factor contributing to ion toxicity for faba bean growing under NaCl salinity

(Geilfus, 2018; Tavakkoli et al., 2010). A critical role for Na^+ appears less relevant, as Na^+ concentrations fluctuated across the varieties at the time point when symptoms occurred (Figs. 6B, 7 B, 9 A). Thus, NaCl tolerance and the ability to withstand salinity (´days without symptoms´) may depend on the ability to restrict the transfer of excess Cl^- to photosynthetically active tissues (Figs. 8C; 9 B). Of note, as Cl^- interferes with NO_3^- uptake an increased NO_3^- selectivity over Cl^-, representing a feature of naturally salt tolerant halophytes, might also be beneficial for glycophytes alongside with avoidance of excess Cl^- uptake (Bazihizina et al., 2018). However, the more tolerant 'Scoop' and 'Nebraska' accumulated fewer Cl^- and therefore might suffered less from excessive ROS production and chlorophyll degradation during the early stress phase than sensitive varieties (Bose et al., 2017; Tavakkoli et al., 2010). Therefore, Cl^- accumulation in the cytosol and further uptake into chloroplasts is expected to be a key factor of ion toxicity in faba bean finally resulting in leaf necrotic spots.

5. Conclusion

In faba bean, ion homeostasis-associated tolerance mechanisms seem to be handled opposingly for Na^+ and Cl^-. Faba bean varieties are tolerant to Na^+ accumulation and consequently Na^+/K^+ ratio is of less importance for evaluating their salt tolerance level. Presumably, tolerance to Na^+ occurred predominantly at the level of tissue tolerance after Na^+ had entered the leaf. Conversely, Cl^- tissue tolerance is weak throughout all 13 *V. faba* varieties as Cl^- concentrations were distinct at the time point of occurring symptoms. Therefore, tolerance to Cl^- was rather facilitated by a restriction of Cl^- entering the plant's shoot. In accordance with the hypothesized Cl^- sensitivity of legumes, Cl^- shoot translocation might be a key process explaining the observed physiological plasticity in the ability to withstand salinity between the diverse *V. faba* varieties.

Conflict of interest

We declare that there are no conflicts of interest.

Authors contribution

BLF, CMG and CZ conceived the study. BLF, MK and XZ conducted the experiments and analysed the data. BLF, CMG and CZ interpreted the data with input from XZ and MK. CZ, CMG and BLF wrote the manuscript. All authors reviewed and approved the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2019.02.012.

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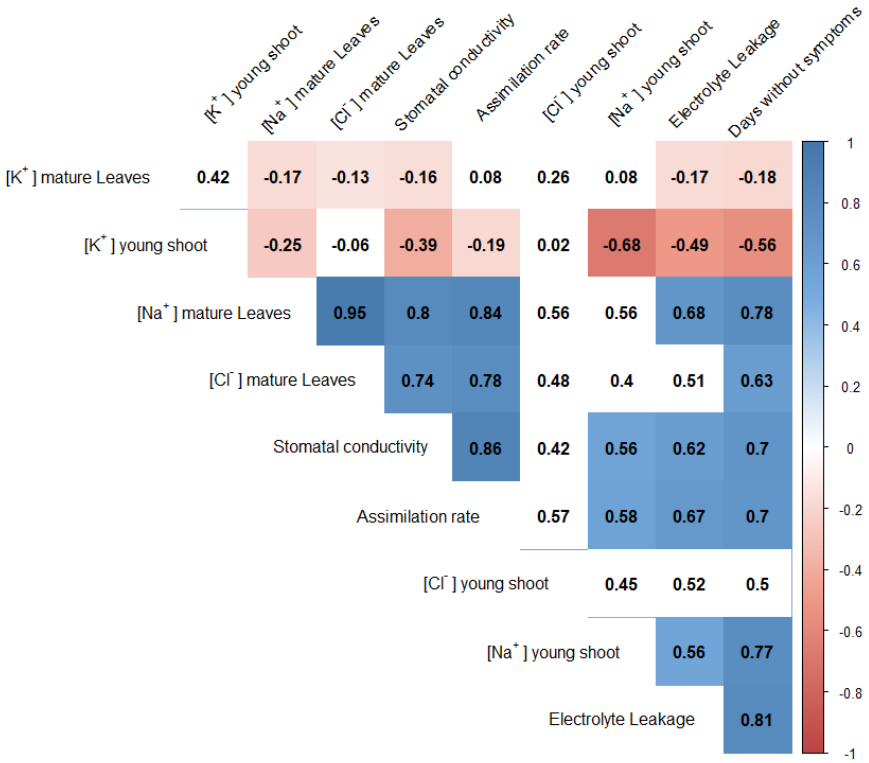
Supplemental materials



Supplementary Figure 1: Correlation matrix of ion concentrations and physiological measurements of 13 salt-stressed *V. faba* varieties (100 mM NaCl). Correlations between concentrations of sodium (Na^+), chloride (Cl^-) and potassium (K^+) in young shoots and mature leaves and electrolyte leakage, days without symptoms and stomatal conductivity. Plant material for ion determination was collected when plants of a variety developed visible salt injuries (see Fig. 2). Stomatal conductivity was measured at mature leaves (4^{th}) 3, 5, 7 and 10 days after full-strength NaCl treatment was applied. Correlations with $p \leq 0.01$ are colour-coded; blue, positive; red, negative.

Supplementary Table 1: Decrease of SPAD value of 13 salt-stressed (100 mM NaCl) V. faba varieties during various stress periods (according to days without symptoms).

***V. faba* L. variety**	**SPAD decrease** [unitless]	**stress period** [d]
‘Fuego‘	-1.9±1.2 C	9
‘Major‘	-5.1±1.2 ABC	9
‘Honey‘	-3.0±1.2 BC	11
‘Mallory‘	-2.9±1.2 BC	11
‘Organdi‘	-2.1±1.2 BC	11
‘Tiffany‘	-1.9±1.2 C	13
‘Diva‘	-3.7±1.2 ABC	16
‘Espresso‘	-2.1±1.2 BC	16
‘Lynx‘	-3.9±1.2 ABC	16
‘RLS5722‘	-8.2±1.2 AB	18
‘RLS67101‘	-9.5±1.2 A	18
‘Nebraska‘	-4.0±1.2 ABC	23
‘Scoop‘	-4.0±1.2 ABC	23

Initial SPAD was measured 3 days after full-strength NaCl stress was applied; Last SPAD measurement was conducted before symptoms occurred. Means from linear model ± SE. Different letters indicate significant inter-variety differences; $p \leq 0.05$; $n = 3$.

8. General discussion

8.1 Responses of maize plants to chloride salinity with respect to crop yield and plant performance

In all experiments (chapter I, II and III), Cl^- was applied as $CaCl_2*2H_2O$ and / or $MgCl_2*6H_2O$, since calcium and magnesium are macronutrients and are not suspected to be toxic at relatively high concentrations (Kirkby and Pilbeam, 1984; Marschner, 2011). Our results show that the Ca concentration and Mg concentration in young maize leaves reach maxima of only 1% and 0.4% respectively, lying in the reported optimal range (Ca: 0.5% to 1.6% DM; Mg: 0.3% to 0.6% DM) (Gaj et al., 2018). Therefore, we consider that the cascade of physiological reactions and effects are attributable to the different applied Cl^- doses.

In general, maize plants are insensitive to Cl^- salinity. All eight genotypes maintained their growth when stressed with 757 mg Cl^- kg^{-1} soil dry matter. Moreover, all cultivars showed unaltered fresh and dry shoot biomass in comparison with the control (Zhang et al., 2019). Even under extreme Cl^- stress (120 mM in nutrient solutions), the genotype P8589, which was moreCl^--sensitive than the others, did not suffer from reduced shoot biomass under the normal supply of NO_3^- (2 mM) (supplemental Fig. S3 of chapter II). In addition to biomass production, the visual toxic appearance provided additional evidence to substantiate the tolerance of maize to high Cl^- concentrations, because only the genotype P8589 exhibited necrosis at the leaf tip and leaf edges of the 9th leaf blade over a period of 10 days after exposure to 757 mg Cl^- kg^{-1} soil dry matter (Zhang et al., 2019). Moreover, the membrane integrity reflected by ion leakage, transpiration rate (E), and stomatal conductance (g_s) of all genotypes were not affected by high Cl^- salinity (Zhang et al., 2019). Although the chlorophyll content indicated by the SPAD value of two genotypes (LG30215 and LG30222) was significantly decreased by 757 mg Cl^- kg^{-1} soil dry matter, it had no inhibition on net photosynthetic rate (A_N) (Zhang et al., 2019), the reason being that

their SPAD readings (50.7 in LG30222 to 54.7 in LG30215) were still higher than the critical threshold of 48.6 fulfilling the photosynthetic function (Sunderman et al., 1997). Even under the extreme conditions of 120 mM Cl^- in the nutrient solution, A_N was not decreased by the reduced g_s and E in the mildly tolerant genotype LG30215 (Fig. 4 of chapter III). One explanation might be that the stomatal regulation predominantly minimized water loss while only marginally inhibiting CO_2 assimilation (Farquhar and Sharkey, 1982).

8.2 Patterns of chloride translocation and tissue storage correlate with tolerance

The two contrasting maize genotypes P8589 and ES-Metronom were able to maintain the Cl^- concentration in shoots at a tolerable level as indicated by the unaltered biomass under 757 mg Cl^- kg^{-1} dry soil (Zhang et al., 2019). One reason might be the ability to restrict the acropetal transport of Cl^- because their translocation factors (TF) < 1, in which TF refers to the ratio of shoot Cl^- concentration to root Cl^- concentration (Zhang et al., 2019 and Fig. 2 of chapter II). A study on *Arabidopsis thaliana* has shown that a reduction in the root-to-shoot transport of Cl^- is an efficient means for reducing the acropetal transport of Cl^- (Li et al., 2016). Moreover, a proportion of the Cl^- that is passed into the shoot is stored in the sheaths of old leaves (Fig. 2 of chapter II). This might be a mechanism for avoiding the accumulation of Cl^- in the blades of old leaves and in younger shoot tissue. In the two fractions of young leaves (the 7^{th} and 8^{th} leaves), the young leaf sheaths showed the higher Cl^- concentration, while the concentration in the blades was always lower (Fig. 3 of chapter II). Therefore, the prevention of Cl^- reaching the site of primary photosynthesis and growing leaves is considered to be a mechanism for coping with Cl^- toxicity (Tavakkoli et al., 2010; Teakle and Tyerman, 2010).

Assuming that plants try to avoid the accumulation of toxic compounds at the site of primary photosynthesis, roots might be a location for storing Cl^- under conditions of Cl^- salinity. An indication for this assumption is derived from one of the genotypes,

namely P8589, in the split-root experiment (Fig. 4 of chapter II). Within the split-root system, roots of P8589 that grew in the chamber that was not supplemented with excess of Cl^- showed a significantly higher Cl^- concentration of 11.6 ± 1.3 mg g^{-1} DM (mean ± SE) than the negative control (3.5 ± 0.5 mg g^{-1} DM) (Fig. 4 of chapter II). Presumably, Cl^- ions were translocated from the side of roots supplemented with 120 mM Cl^- to the other side of roots without excess Cl^- within the same plant growing in the split-root device. This phenomenon was confirmed under experimental condition whereby Cl^- uptake was reduced. Such conditions were implemented by inducing uptake competition between Cl^- and NO_3^-, *i.e.*, by increasing the NO_3^- concentration in the nutrient solution from 2 mM to 8 mM (Fig. 5 of chapter II).

Storage of Cl^- in roots might be an effective means for diluting the tissue concentration of Cl^- in order to avoid thresholds being exceeded. However, whether such Cl^- movement was the result of a root-to-root translocation mechanism or of a re-translocation of Cl^- from the shoot back to the root in the split root experiment remained unclear. In our work, we only observed Cl^- movement from roots that were supplemented with excess Cl^- into the other roots without excess Cl^- in the genotype P8589; thus, a generalization of this effect was not possible. The leaf-brushing experiment with Cl^- gave the answer that Cl^- movement in roots was the result of root-to-root translocation rather than the result of shoot-to-root re-translocation: Cl^- brushed onto the 5th leaf blade did not increase the Cl^- accumulation in roots, although it significantly elevated the Cl^- concentration of old leaves (blades and sheaths) (Fig. 6 of chapter II). A future task will be to gather understanding about the way how Cl^- moves from root to root. The symplastic route would require a protein facilitated cellular uptake. We assume that a transfer via the apoplastic route is also possible.

In brief, tolerance to Cl^- salinity in maize primarily comes from the restricted acropetal transfer of Cl^- and the preferable accumulation away from the primary site of photosynthesis and growth. Moreover, Cl^- movement in roots might be helpful in diluting the root Cl^- concentration.

8.3 Effects of osmotic stress and chloride stress on biomass and chlorophyll content

The osmotic stress phase dominates more with regard to the reduced plant biomass than to Cl^- ion toxicity. No evidence supports the direct inhibition of Cl^- toxicity on the shoot biomass of the mildly tolerant genotype LG30215. First, PEG-induced osmotic stress causes the biomass reduction of shoot as strongly as stress with 120 mM Cl^-, whereas PEG only produces osmotic pressure accounting for 57 % of that induced by 120 mM Cl^- (Fig. 2 of chapter III). Second, the visual appearance of small necrotic symptoms on older leaves simultaneously emerges under both high Cl^- stress and PEG-induced osmotic treatment (Fig. 2 of chapter III). Last but not least, the PCA pattern (Fig. 5 of chapter III) indicates that plant biomass and PEG-dependent osmotic stress appear to be highly negatively correlated (arrows in completely the opposite direction). The growth reduction of maize under NaCl stress has been reported to be strongly related to the osmotic phase effects (Cramer et al., 1994; De Costa et al., 2007). Thus, the osmotic-stress-induced growth inhibition in maize is not specific to Cl^-.

Stress-induced reductions of the chlorophyll content in maize is attributable more to osmotic phase than to the Cl^--specific toxicity phase. Osmotic imbalance leads to a chlorophyll content reduction of 32.6% under PEG-based osmotic treatment, and an obviously negative correlation exists between SPAD and osmotic stress clustered by PCA plots (Fig. 4A and Fig. 5 of chapter III). This agrees with the previous report that water stress is able to reduce chlorophyll content by up to 40% without any interference of photosynthesis at mid-day in maize (Sanchez et al., 1983). Moreover, chlorophyll loss is thought to be independent of applied Cl^- dose (Fig. 4A of chapter III). This has also been observed in soil-cultivated maize (Zhang et al., 2019). Therefore, the Cl^--stress-related reduction of chlorophyll in maize results from the osmotic component rather than from Cl^- ionic toxicity.

Overall, the osmotic phase of Cl^- salinity plays a more important role in growth inhibition and the reduction in chlorophyll content.

8.4 Effects of osmotic stress and chloride stress on nitrate reductase activity

The mechanism of nitrate reductase activity (NRA) interference between PEG-induced osmotic stress and Cl^- stress is different. Upon PEG-induced osmotic stress (plus 4 mM Cl^-), the osmotic imbalance possibly causes more Cl^- to enter into the leaf cells (Fig. 3A of chapter III). Apart from acting as a `cheap´ osmoticum (Wege et al., 2017), Cl^- is also thought to liberate (by means of substitution) other osmotica such as proline, sucrose, malate, K^+, and NO_3^- for use in other functions (Flowers, 1988). The lower NO_3^- concentration is also speculated to result from decelerated mass flow, because E is significantly reduced by PEG-induced osmotic stress (Fig. 4C of chapter III). Interestingly, the presence of less NO_3^- in the fully developed leaf blade does not disturb NO_3^- metabolism, as NRA is not affected by PEG-osmotic stress (Fig. 3B of chapter III).

By contrast, the inhibition of NRA by Cl^- stress is highly related to Cl^- and NO_3^- uptake competition (Fig. 3 of chapter III). The root-located ion antagonism possibly impacts on NO_3^- uptake because of the presence of excessive Cl^- in the external medium; this might affect NRA, as a shortage of NO_3^- in leaf tissues has previously been reported greatly to undermine NRA (Flores et al., 2000). In order to overcome this Cl^- / NO_3^- uptake competition in salt adversity, genetic modifications have been conducted, *e.g.*, the overexpression of chloride channels (GmCLC1, GsCLC-c2) in hairy roots of soybean has been found to enhance salt tolerance. The over-expressed transport proteins are highly correlated with the significant decrease of Cl^- content in shoots and then indirectly maintain a high NO_3^- accumulation in plant tissues, thereby keeping a constant lower ratio of Cl^- / NO_3^- in the stems and leaves of soybean (Wei et al., 2019; Wei et al., 2016). However, this might not be sufficient to guarantee the metabolic function of NR under saline conditions, because the osmotic component of salt stress has consistent slowing-down effects on mass flow. If the stress is strong enough, the decelerated mass flow would considerably decrease the transport

of NO_3^- to the leaf tissues. As a consequence, the scarcity of NO_3^- in the metabolic tissues would inevitably limit NRA, irrespective of any Cl^- and NO_3^- uptake competition at the roots.

In summary, osmotic stress does not interfere with NRA but slows down mass flow, which probably reduces NO_3^- transport to the leaf tissues, whereas excess Cl^- indirectly inhibits NRA through the antagonistic limitation of NO_3^- uptake.

8.5 Comparison of sensitivity to chloride salinity between maize and faba bean

In comparison with maize plants, legumes, in particular the faba bean, are more sensitive to high NaCl stress (Li et al., 2017b; Tavakkoli et al., 2010). Although Na^+ toxicity is widely studied, the toxic aspects of Cl^- and the underlying tolerance mechanisms to Cl^- are however not well understood (Franzisky et al., 2019). The present work indicates that responses to Cl^- stress greatly differ between these two species.

Mature fully expanding leaves of faba bean are more likely to be vulnerable to a high tissue Cl^- concentration than those of maize plants. The evidence is that almost all 13 faba bean varieties, except for the tolerant variety Major, exhibit serious necrotic lesions of the 4th leaf after exposure to 100 mM NaCl, when the concentration of Cl^- is in the same range (100-420 µmol g^{-1} DM, equivalent to 3.5-14.9 mg g^{-1} DM), irrespective of the genotype (Franzisky et al., 2019). In contrast, given that maize suffers from the same range of Cl^- (7.7-14.8 mg g^{-1} DM) in leaf tissues, only genotype P8589, which is the most sensitive to Cl^- out of 8 tested cultivars, shows slight chlorotic symptoms at the leaf edges and leaf tip on the 9th leaf blade (Zhang et al., 2019). The symptomatic comparison of these two species suggests that maize is able to withstand more Cl^- than faba bean plants. In addition, the quantified Cl^- (relatively stable) and Na^+ (considerably fluctuating) in the leaf tissues of faba bean at the time-point of necrosis appearance indicates that Cl^- concentrations rather than Na^+ concentrations are critical for the formation of salt necrosis in faba bean plants.

Therefore, the sensitivity of faba bean to Cl^- stress plays a more important role than that of Na^+ in contribution to the sensitivity to NaCl salinity.

Regardless of species, the genotypic differences to Cl^- stress is hypothesized to be primarily associated with the ability to exclude from shoots. In faba bean, restricted root-to-shoot Cl^- translocation is regarded as a potential determinant for Cl^- tolerance, since tolerant varieties benefited from lower Cl^- translocation to the leaves (Franzisky et al., 2019). For instance, the more tolerant genotypes Scoop and Nebraska accumulate less Cl^- in leaf tissues (Franzisky et al., 2019). In parallel, the maize genotype ES-Metronom, which is more tolerant to Cl^- maintains a significant lower level of Cl^- in leaf blades than the genotype P8589, which is more sensitive to Cl^- (Fig. 2 and Fig. 3 of chapter II). This is because that the genotype ES-Metronom has the strong ability to exclude Cl^- from the shoot because of its lower TF than that of the genotype P8589 (Fig. 2A of chapter II).

To sum up, maize is more tolerant to Cl^- salinity than faba bean. The restriction of Cl^- root-to-shoot translocation is a key trait of Cl^- tolerance in both crops.

8.6 Outlook

(1) Maize possesses the strong ability to withstand Cl^- salinity. This is achieved by the restriction of Cl^- transfer from the root to shoot and also the preferable sequestration of Cl^- away from young photosynthetic sites. Both mechanisms are implemented by Cl^- stress-induced gene, protein, and metabolic regulation mechanisms. Thus, the screening of key responsive genes might be a suitable way of engineering gene-edited crops with a better performance under Cl^- salinity.

(2) Crop growth is partly affected by nitrogen use efficiency (NUE) including NO_3^- uptake and NO_3^- metabolism. These processes are influenced by the osmotic and ionic components of Cl^- salinity. Therefore, NUE will be optimized only if NO_3^- uptake from the root to shoot and NO_3^- metabolism in the leaves are increased or at least maintained under Cl^- salinity. Engineering of the key Cl^- / NO_3^- transporters such as chloride channel CLCs is able to maintain a high accumulation of NO_3^- under

conditions of Cl^- salinity. Moreover, the strategy of trying to minimize the sensitivity of stomata to osmotic stress or Cl^- toxicity should be also considered, because stomatal closure-triggered transpiration reduction can slow down mass flow, finally decreasing NO_3^- transfer.

(3) The split-root experiments have shown that Cl^- movement from roots supplemented with excess Cl^- to the other roots without excess Cl^- within the same plant exists and might function in Cl^- dilution. This mechanism should be further validated by tracing ^{36}Cl, as it has not been observed in all genotypes. Moreover, these findings derive from hydroponic solution culture, and therefore, whether they will appear under field conditions remains unclear.

Despite this, the present split-root experiment results raise further questions of interest:

i. How do roots sense the lack of Cl^-?

ii. Which signals are triggered to facilitate the uptake and transfer of Cl^- from roots supplemented with excess Cl^- back to the other roots lacking Cl^- in, for example, the split-root device?

iii. Which key Cl^- / NO_3^- transporters are responsive to signal regulation?

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Affidavit

Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

Translocation and storage of chloride in chlorine-stressed maize (Zea mays L.)

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Stuttgart, 16.06.2020 — Place, Date

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