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Effects of the substitution of potassium by sodium on physiological processes and their responsible key enzymes in sugar beet

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Submitted by

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To my family

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1 Introduction

“Each week the world loses an area larger than Manhattan to salt-degradation.”

(Adeel cited by United Nations University, 2017)

This statement illustrates the significance of the increasing problem of soil degradation by salts. Soil degradation affects food production and contributes to the fact that almost 800 million humans worldwide suffer chronic hunger (FAO, 2015a). Worldwide about 1 billion hectares of soil are salt-affected (FAO, 2015b). Soil degradation by salinity and sodicity threatens not only food security of humans and animals in countries, whose economies are mainly based on agriculture, but the salinity-caused shortage of food can lead to civil disturbances. These disturbances contribute to political instability such as 2011 in Syria, which further worsen the living conditions of the population (Gleick, 2014).

1.1 Soil salinity

Salt-affected soils are impaired by salinity and/or sodicity. Soils are classified as saline, when the electrical conductivity (EC) of the saturated paste extract is higher than 4 dS m^{-1}, which causes growth reductions of most crop species (United States Salinity Laboratory Staff, 1954). Sodic soils exhibit an exchangeable sodium percentage (ESP) higher than 15 (Qadir et al., 2007). Saline sodic soils, which comprise both problems, are characterized by an EC higher than 4 dS m^{-1} and an ESP higher than 15 (Schubert, 2015).

Soil salinity is a global problem, especially in arid and semi-arid regions. In these climate regions evaporation exceeds precipitation. Due to evaporation the concentration of ions in the soil solution increases. Insufficient precipitation does not allow leaching of salts. Ascending water transports salts from deeper soil layers upwards in the soil profile. Salts accumulate in top layers and crystallize at the soil surface (Schubert, 2015). The intensity of this natural primary salinity is increased in vicinities of seas: Oceanic salts carried by rain and wind increase the salt load near coasts (Munns and Tester, 2008). Moreover, soluble salts released from parental rocks promote the accumulation of salts in upper soil layers. Soluble salts resulting from weathering of parental rocks are mainly chlorides of sodium, calcium, and magnesium and, to a lesser extent, sulfates and carbonates (Szabolcs, 1994).

In addition to primary salinity, which is caused by natural factors, incorrect cultivation techniques lead to secondary salinity. When water with dissolved salts is used for irrigation, these additionally supplied salts accumulate after evaporation of the water in soil (Schubert, 2011; Szabolcs, 1994). Also poor drainage facilities or continuous irrigation over long periods are responsible for human-induced soil degradation (FAO, 2015b).

1.2 Salt stress of plants

Plant growth is inhibited indirectly and directly by soil salinity. Plant growth is indirectly impaired by the degradation of the soil structure. Sodium ions (Na^+) compete with calcium ions (Ca^{2+}) for binding sites on soil particles. The Na^+ cannot build bridges to soil particles such as Ca^{2+}. The cohesion between the soil particles is disturbed by the large hydration shell of sodium ions; hence, Na^+ shows a peptizing effect on soil structure and the stability of soil aggregates is lost. The water and air supply of the soil is thereby impaired. Therefore, rooting, root respiration, and uptake of nutrients are disturbed (Qadir et al., 2007; Schubert, 2011).

1.3 Three-phase model of salt stress

Plant growth is also directly inhibited by soil salinity. The response of plants to salt stress can be described by the two-phase model of Munns (1993). This conceptual model differentiates the osmotic and ionic effects of salt stress on plant growth (Munns, 1993). However, the two-phase model excludes short-term effects of salt stress (Schubert, 2011). This immediate response of the plants to salt stress was integrated in the model by Schubert (2011). Therefore, the model was extended to a three-phase model by adding one more phase, which describes the short-term effects of salt stress (Phase 0) (Figure 1).

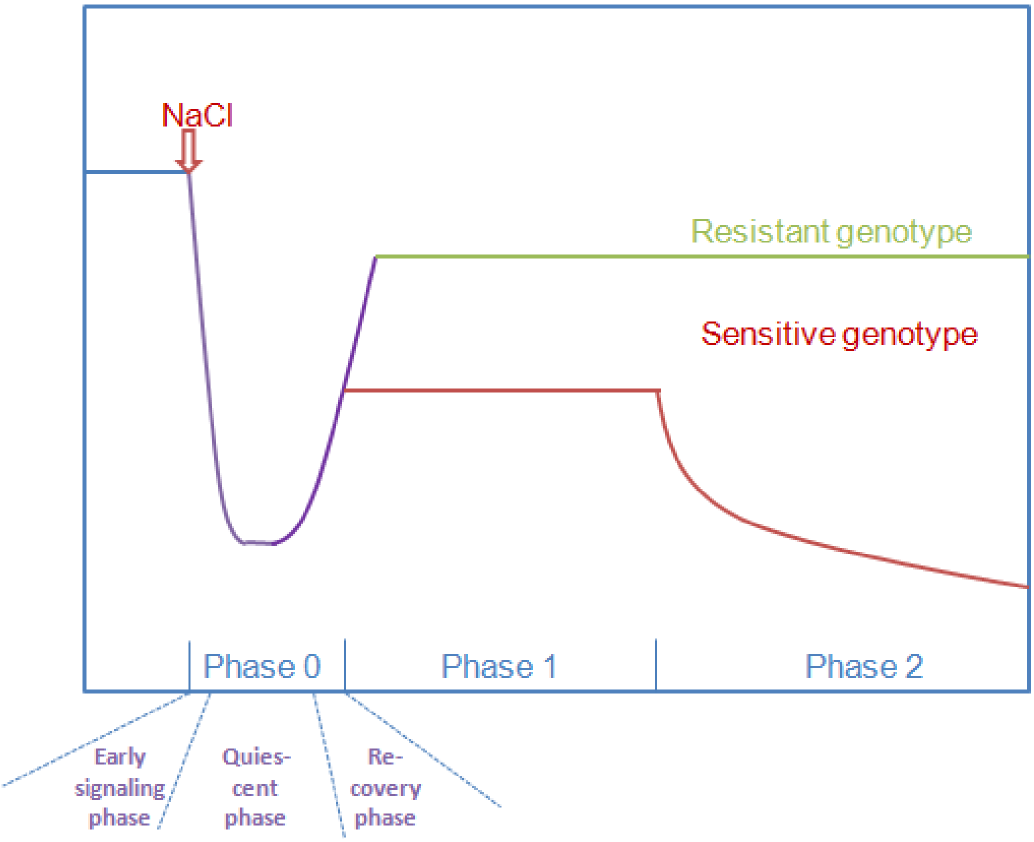

Figure 1: Modified model of the plant response to salt stress according to Munns (1993), Schubert (2011), and Julkowska and Testerink (2015). Phase 0 includes short-term effects of salt stress on plant growth. Phase 1 is characterized by osmotic stress, while plants are affected by ion toxicity in Phase 2. The red line illustrates the response of a genotype which is sensitive in Phase 1 and 2. The green line shows a genotype which is partially resistant in Phase 1 and fully resistant in Phase 2.

1.3.1 Phase 0

Phase 0 starts immediately after the supply of sodium chloride (Figure 1). Entry of Na^+ into the root cells via nonselective cation channels (*NSCC*) and members of the high-affinity potassium transporter (*HKT*) family leads to depolarization of the membrane potential of root cells (Garciadeblás et al., 2003; Demidchik and Maathuis, 2007). Moreover, plants suffer reduced water availability and turgor of plants is reduced in Phase 0. Sometimes wilting of plants is observed (Schubert, 2011).

Epidermal and cortical root cells sense the ionic and osmotic components of salt stress (Munns and Tester, 2008) and induce local signaling (Cheeseman, 2013). Transient membrane depolarization, which is caused by Na^+ influx, and turgor loss, which is sensed by mechano- and osmosensitive receptors, lead to NaCl stress-specific increase in cytosolic Ca^{2+} concentration and reactive oxygen species (ROS) production within seconds to few minutes after exposure to salt stress (Munns and Tester, 2008; Julkowska and Testerink, 2015). The Ca^{2+} signal and elevated ROS levels induce abscisic acid (ABA) synthesis within hours. ABA initiates the activation of transcriptional responses (Julkowska and Testerink, 2015). There are not only local signaling cascades in sensing root cells, but also systemic signaling ones which induce integrated plant-wide responses (Cheeseman, 2013). However, the precise nature and components of systemic signaling are not clear. This long-distance signaling is mediated at least partly by ABA (Munns and Tester, 2008). Besides ABA, gibberellic acid (GA), ethylene, and jasmonate are involved in the interconnected salt-activated signaling network (Achard et al., 2006; Golldack et al., 2014). Also Ca^{2+} waves are likely to contribute to the long-distance root-to-shoot signaling under saline conditions (Choi et al., 2014).

This early signaling response is followed by a quiescent phase (Figure 1) which is characterized by maintenance of a low growth rate (Deinlein et al., 2014; Julkowska and Testerink, 2015; Munns, 2002). Growth is reduced by the inhibition of cell division and cell extension. Cell division is inhibited due to ABA-mediated stabilization of DELLA proteins (Achard et al., 2006) which inhibit the growth-promoting action of GA (Ueguchi-Tanaka et al., 2007). Extension growth is reduced by the decrease of turgor and cell-wall extensibility, which may be caused by ROS-mediated crosslinking of phenolic compounds and glycoproteins in the apoplast (Julkowska and Testerink, 2015; Tenhaken, 2015).

However, these changes in growth rate, membrane potential, and turgor are transient and recover after some time (Läuchli and Schubert, 1989; Thiel et al., 1988). In this recovery phase (Figure 1) turgor is restored by the sequestration of Na^+ in vacuoles. Restored turgor allows turgor-driven extension growth (Julkowska and Testerink, 2015). Moreover, extension growth is promoted by an increase in cell-wall extensibility. This increase in cell-wall extensibility could be explained with an excess of ROS (especially of OH·) over cross-linkable cell-wall compounds, which leads to the cleavage of cell-wall polysaccharides (Tenhaken, 2015). Growth recovery is also supported by activation of GA biosynthesis which enables reactivation of cell division (Yan et al., 2014). The extent of recovery depends on the sensitivity of early signaling and the capacity for acclimation (Munns and Tester, 2008; Julkowska and Testerink, 2015). Due to its transient character Phase 0 is not physiologically relevant nor can it be used for breeding purposes. But it is important to avoid this phase when physiological experiments are carried out (Schubert, 2011).

1.3.2 Phase 1

Although growth recovers, the growth rate of not fully resistant plants does not reach the level of unstressed plants. Plants in this phase of salt stress (Phase 1) suffer osmotic stress (Figure 1) (Munns, 1993; Schubert, 2011). The lowered water availability imposes water stress on the plants. However, plants are adapted to the reduced water availability and do not suffer water stress due to osmotic adjustment. This is supported by the maintenance of turgor in Phase 1 (De Costa et al., 2007) and the absence of wilting symptoms (Schubert, 2011). Cell extension and cell division of sensitive plants are inhibited (Munns and Tester, 2008). Especially shoot growth is strongly reduced in the first phase of salt stress (Fortmeier and Schubert, 1995; Zörb et al., 2005; Hatzig et al., 2010; Hütsch et al., 2016). Therefore, plants show stunted growth and are sometimes dark-green color in Phase 1 (Schubert, 2011). Moreover, osmotic stress can induce physiological calcium deficiency (Fortmeier and Schubert, 1995; Tavakkoli et al., 2010; Schubert, 2011). It was shown that genotypic differences exist also in the first phase of salt stress (Schubert et al., 2009; Schubert, 2011). Plants resistant in Phase 1 show a better growth performance than sensitive plants (Figure 1). Genotypic differences in salt resistance in Phase 1 can be partly ascribed to a different extent of apoplastic acidification in maize leaves (Zörb et al., 2005;Pitann et al., 2009). According to the acid-growth theory apoplastic acidification by plasma membrane (PM) H^+-ATPase increases cell-wall extensibility and allows turgor-driven extension growth (Hager et al., 1971; Hager, 2003). Genotypic differences in apoplastic acidification may rely on different sensitivity of phytohormonal signaling, on the differential expression of efficient isoforms or on posttranslational modifications of PM H^+-ATPase in Phase 1 of salt stress (Zörb et al., 2005; Pitann et al., 2009; Naeem, 2011). Genotypic differences also exist in the composition of cell walls and development of cell-wall maturation of maize leaves (Eitenmüller, 2016; Uddin et al., 2013). These differences affect cell-wall extensibility and, hence, the growth of maize leaves (Eitenmüller, 2016).

1.3.3 Phase 2

However, osmotic stress is not the only problem that arises from salt stress. Phase 1 is followed by Phase 2 that causes further growth reductions (Figure 1) (Munns, 1993). Due to high concentrations of salts in soil or nutrient solution plants cannot avoid a gradual accumulation of ions of these salts. This high cytoplasmic accumulation of ions leads to the disturbance of plant metabolism. Toxicity symptoms (chlorosis, necrosis) are observed firstly on old leaves, since they are exposed longest to the transpiration stream and maturation of old leaves does not allow diluting of incoming ions (Munns, 1993; Munns and Tester, 2008). Especially Na^+ and chloride (Cl^-) can accumulate to toxic levels (Munns, 1993; Fortmeier and Schubert, 1995). The accumulation of Na^+ also induces a deficiency of potassium. Whereas Cl^- is considered to be the more harmful ion for lemon, soybean, and grapevine (Munns and Tester, 2008), it was shown that Na^+ is the most toxic ion in Phase 2 for most crops e.g. maize (Fortmeier and Schubert, 1995). Sümer et al. (2004) showed that effects of ion toxicity can also occur in the first phase of salt stress. However, the effects are quantitative negligible (Schubert, 2011). It was shown that genotypic differences in salt resistance of Phase 1 and 2 exist (Figure 1) (Munns, 1993; Pitann et al., 2013). Therefore, the Phases 1 and 2 are physiologically relevant and important for breeding purposes. The combination of resistance traits of Phase 1 and 2 in salt-resistant (SR) maize hybrids was successfully achieved by conventional breeding methods (Schubert et al., 2009).

1.4 Substitution of potassium by sodium

Although the “twins” K^+ and Na^+ (Benito et al., 2014) show many similarities, their role in plant nutrition differs. Whereas K^+ is a macronutrient of all higher plants, Na^+ is only essential in traces for some halophytes and C4 plants (Adams and Shin, 2014). As a macronutrient potassium fulfills three key functions in plants: Charge balance, osmotic functions, and enzyme activation. Due to the large diameter of the hydrated Na^+ these key functions of potassium cannot completely be fulfilled by sodium (Schubert, 2015). Especially specific functions of K^+ such as enzyme activation cannot be taken over by Na^+ (Evans and Wildes, 1972). Hence, it is important for cells to maintain K^+ homeostasis in the cytoplasm under salt stress (Anschütz et al., 2014).

The accumulation of Na^+ and the displacement of K^+, which occur in Phase 2 of salt stress, can be simulated by the cultivation of plants in nutrient solution in which K^+ is substituted by Na^+. By using this experimental approach, the replaceability of K^+ by Na^+ can be tested by avoiding Phase 1 effects. By keeping the sum of supplied K^+ and Na^+ constant at the level of optimal K^+ supply, additional effects caused by osmotic stress are deliberately excluded (Faust and Schubert, 2016, 2017).

It is well known that K^+ can be replaced by Na^+ to a great extent in *Beta vulgaris* (Troug et al., 1953). However, the possible extent of substitution of K^+ by Na^+ is also limited in sugar beet. It was not clear which process is most sensitive towards the substitution of K^+ by Na^+ and, therefore, limits the extent of substitution (Faust and Schubert, 2016). Hence, one aim of this thesis was to identify the process which is most sensitive to the substitution of K^+ by Na^+ in sugar beet.

Based on findings in the literature, we identified the processes of transpiration, growth, and net protein synthesis as possible processes which could suffer from the substitution. Hence, the following hypotheses (Faust and Schubert, 2016) were set up and tested in a substitution approach:

1. K^+ is important for opening and closing of stomata. Na^+ disrupted the ability of stomatal closure in *Vicia faba* (Slabu et al., 2009). It is hypothesized that transpiration is most sensitive to the substitution of K^+ by Na^+ (Faust and Schubert, 2016).

2. K^+ fulfills many functions in the process of plant growth (Hawkesford et al., 2012). It is hypothesized that the growth of young sugar beet plants is most sensitive to the substitution of K^+ by Na^+ (Faust and Schubert, 2016).

3. K^+ deficiency reduced significantly the protein content of maize (Hsiao et al., 1969). It is hypothesized that protein synthesis is most sensitive to the substitution of K^+ by Na^+ (Faust and Schubert, 2016).

The first study of this thesis (Faust and Schubert, 2016) showed that net protein synthesis is the most sensitive process under substitution of K^+ by Na^+. However, net protein synthesis was not inhibited due to substrate limitation (Faust and Schubert, 2016). Therefore, the following hypothesis was set up and tested in an *in vitro* approach (Faust and Schubert, 2017):

4. It is hypothesized that the process of translation itself is inhibited due to the substitution of K^+ by Na^+ (Faust and Schubert, 2017).

The observed sensitivity of protein synthesis towards the substitution of K^+ by Na^+ (Faust and Schubert, 2017, 2016) underlines the importance of avoidance of Na^+ accumulation and maintenance of a high K^+/Na^+ ratio in the cytoplasm (Blumwald et al., 2000). There are two strategies for maintenance of ion homeostasis: minimizing the entry of Na^+ (exclusion) and sequestration of Na^+ in vacuoles (inclusion) (Munns, 2002; Schubert, 2015).

The strategy to keep the entry of Na^+ into the plant as low as possible is achieved by passive and active exclusion of Na^+ at the root surface (Greenway and Munns, 1980; Schubert, 2015). Passive exclusion of Na^+ is achieved by ion channels that discriminate K^+ and Na^+ and do not allow Na^+ to pass. It was shown that low passive Na^+ permeability leads to reduced Na^+ influx rates in maize (Schubert and Läuchli, 1990). However, it is not clear which root cell types (epidermal, cortical, or endodermal) enable selectivity (Munns, 2002). In addition to passive exclusion of Na^+ due to the specificity of cation channels, the casparian band prevents the unselective entry of ions into the root stele. However, this endodermal barrier is not perfect. The casparian band is interrupted at the sites of secondary root formation and in the apical region in some plant species (Robards and Jackson, 1976; Peterson et al., 1981; Faiyue et al., 2010). It was shown that apoplastic bypass flow across the endodermis can contribute significantly to the amount of Na^+ which enters the xylem under saline conditions (Yeo et al., 1987). Krishnamurthy et al. (2009) showed that an increased thickness of the casparian band, which blocks the bypass flow of water and solutes, contributes to the salt-resistance of some rice genotypes.

A further possibility to avoid Na^+ accumulation is the export of Na^+ taken up. The only transporter enabling active Na^+ efflux is the Na^+/H^+ antiporter Salt Overly Sensitive 1 (*SOS1*). The pH gradient necessary for Na^+ export is provided by PM H^+-ATPase. However, the precise function of this PM-located antiporter and its physiological significance contributing to Na^+ exclusion under saline conditions are not fully understood (Britto and Kronzucker, 2015). Since *SOS1* activity is dependent on the pH gradient established by PM H^+-ATPase, the contribution of *SOS1* to Na^+ extrusion in alkaline saline soil is supposed to be low due to the lack of H^+ (Schubert, 2015; Falhof et al., 2016; Hamam et al., 2016). Experiments of Kiegle and Bisson (1996) demonstrated that Na^+/H^+-antiport systems contribute to Na^+ efflux in *Chara* algae at pH 5-7, but not at pH 9. Whereas *SOS1* is highly expressed in parenchyma cells at the xylem/symplast boundary of roots, stems, and leaves and seems to be able to retrieve Na^+ from the xylem sap depending on severity of salt stress (Shi et al., 2002), expression of *SOS1* in roots is reported only for cells of root tips (Shi et al., 2002). Also experiments of Hamam et al. (2016) support the role of *SOS1* in the export Na^+ from distal regions of the root, and not from the bulk root. Since meristematic cells do not have the possibility to sequester Na^+ in vacuoles, the activity of *SOS1* could be necessary to maintain a high K^+/Na^+ in the cytosol of root-tip cells (Shi et al., 2002; Britto and Kronzucker, 2015).

A possibility to allow Na^+ export under alkaline conditions would be the export of Na^+ by Na^+-ATPases. The presence of Na^+-ATPases has been suggested for algae (Blumwald et al., 2000). The marina alga *Heterosigma akashiwo* possess a Na^+-ATPase (*HANA*) which shows similarities with the Na^+/K^+-ATPase in animals. *HANA* enables the alga to maintain a low intracellular Na^+ concentration in its saline habitat (Wada et al., 1989, 1992; Shono et al., 2001). The presence of an Na^+-ATPase, which shows similarity with fungal *ENA* (exitus natrium)-type Na^+-ATPases, was identified in the moss *Physcomitrella patens* (Benito and Rodríguez-Navarro, 2003). However, Na^+ pumps have not (yet) been identified in higher plants.

The second strategy to maintain a high K^+/Na^+ ratio in the cytoplasm is the exclusion of Na^+ from cytoplasm by sequestration of Na^+ in vacuoles. This strategy is used efficiently by halophytes (Munns, 2002). Sodium is sequestered in vacuoles by means of Na^+/H^+-antiporter *NHX* in the tonoplast (Adler et al., 2010). Na^+ efflux from vacuole into cytoplasm is prevented by efficient control of slow (SV) and fast (FV) vacuolar channels in the halophyte *Chenopodium quinoa* (Bonales-Alatorre et al., 2013).

The successful compartmentalization of Na^+ (and Cl^-) is responsible for the increased salt resistance of halophytes compared to glycophytes (Greenway and Munns, 1980; Munns, 2002). This was supported by the findings of Flowers (1972) and Greenway and Osmond (1972). They showed that cytosolic enzymes from halophytes exhibited a similar sensitivity to high Na^+ concentrations as those from glycophytes. The tested enzymes from halophytes did not show a higher tolerance to elevated Na^+ concentrations. These findings led Greenway and Munns (1980) to the postulation of the dogma that the basis for the salt resistance of halophytes is the successful compartmentalization of Na^+ and that halophytic enzymes are not adapted to high Na^+ concentrations.

More than thirty years later, we questioned whether this dogma is still valid. Reason for the doubt of validity of the dogma was provided by the results of Wakeel et al. (2011). These authors showed that the key enzyme PM H^+ -ATPase of sugar beet is significantly less susceptible to the substitution of Na^+ by K^+ than the key enzyme from maize. Based on the results of Wakeel et al. (2011), the following hypothesis was set up (Faust and Schubert, 2017):

> 5. It is hypothesized that the activity of sugar beet ribosomes, which also play key role in the plant organism, are less susceptible to the substitution of K^+ by Na^+ than the ribosomes of maize (Faust and Schubert, 2017).

2 Publication 1: Protein synthesis is the most sensitive process when potassium is substituted by sodium in the nutrition of sugar beet (*Beta vulgaris*)*

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Research article

Protein synthesis is the most sensitive process when potassium is substituted by sodium in the nutrition of sugar beet (*Beta vulgaris*)

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A B S T R A C T

Potassium ions (K^+) and sodium ions (Na^+) share many physical and chemical similarities. However, their interchangeability in plant nutrition is restricted. Substitution studies showed that K^+ can be replaced by Na^+ to a large extent in the nutrition of *Beta vulgaris* L. However, the extent of substitution without negative impacts is not unlimited. The aim of the present study was to identify the process which is most sensitive during the substitution of K^+ by Na^+ in nutrition of young sugar beet plants. We focused on transpiration, growth, and net protein synthesis. Plants were grown under controlled environmental conditions. With transfer of seedlings into nutrient solution, plants were cultivated in different substitution treatments. For all treatments the sum of K^+ and Na^+ (applied as chloride) was fixed to 4 mM. The extent of substitution of K^+ by Na^+ in the nutrient solution was varied from low (0.25% substitution: 3.99 mM K^+, 0.01 mM Na^+) to almost complete substitution (99.75% substitution: 0.01 mM K^+, 3.99 mM Na^+). The supply of 3.99 mM K^+ in 0.25% substitution treatment guaranteed the absence of K^+ deficiency. Transpiration was not affected by the substitution. Growth was inhibited at a substitution level of 99.75%. Net protein synthesis was already affected at a substitution level of 97.50% (0.10 mM K^+, 3.90 mM Na^+). Hence, net protein synthesis was most sensitive to the substitution and limited the extent of substitution of K^+ by Na^+ in the nutrition of young sugar beet plants.

1. Introduction

Potassium ions (K^+) and sodium ions (Na^+) are present at high concentrations in the earth crust. Although these two monovalent inorganic cations share many similarities, their exchangeability in plant nutrition is restricted (Benito et al., 2014). Key functions of K^+ in plants are charge balance, enzyme activation, and osmotic functions. These specific functions of K^+ cannot be completely fulfilled by Na^+ due to its lager hydration shell compared to K^+ (Schubert, 2015). The hydrated Na^+ is not able to pass cation channels which often possess a high specificity for K^+, and therefore the permeability of Na^+ is too low to accomplish charge balance. Activation of many enzymes by K^+ is based on the sorption of K^+ to enzymes, which leads to conformational changes allowing stimulation of enzyme activity. Due to its larger radius of the hydrated Na^+, binding energy to enzymes is much lower compared to that of K^+ and does not allow conformational changes required for optimal enzyme activity. Although unspecific osmotic functions of K^+ can be taken over by Na^+, the low membrane permeability of Na^+ prevents that the regulation of the water economy can be fully guaranteed by Na^+ (Schubert, 2015). Due to the various key functions of K^+ the regulation of K^+ homoeostasis has been described as an essential process of plant adaptive responses to the environment (Anschütz et al., 2014).

The possible extent of substitution of K^+ by Na^+ varies strongly among plant species. It has been known for many years that K^+ can be replaced by Na^+ to a great extent in *Beta vulgaris*. These studies can be classified in three different approaches which focus on completely different aspects of the relationship between K^+ and Na^+ in the nutrition of *Beta vulgaris* under non-saline conditions (Fig. 1). Studies dealing exclusively with the response of *Beta vulgaris* to salt stress are not considered here because the interaction of K^+ and Na^+ could be masked by effects caused by osmotic stress or ion toxicity induced by salinity. Many authors studied the effect of adding Na^+ in the presence of adequate amounts of K^+ on growth and physiology of beets (e.g. Milford et al., 2008) (Fig. 1, Approach 1). In a second approach, it was investigated whether Na^+ can

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Abbreviations: PM, plasma membrane; PPFD, photosynthetic photon flux density; DAS, days after sowing; DM, dry matter; SE, standard error.

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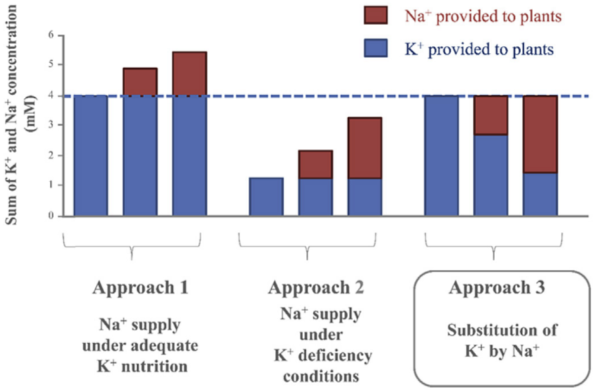

Fig. 1. Schematic illustration of experimental approaches dealing with the interaction of K^+ and Na^+ under non-saline conditions. Horizontal line indicates the level of adequate K^+ concentration.

restore growth, morphology, and physiology of beets deficient in K^+ (e.g. Subbarao et al., 2001) (Fig. 1, Approach 2). Although some experiments following the Approach 1 and/or Approach 2 were titled as substitution studies, we consider studies following the Approach 3 as substitution studies (e.g. Marschner et al., 1981) (Fig. 1, Approach 3). Only this third approach, in which the sum of supplied K^+ and Na^+ concentrations is kept constant equal at the level of adequate K^+ nutrition, can give reliable information about the replaceability of K^+ by Na^+ in the nutrition of beet.

Moreover, the onset of the substitution treatment has to be considered. Due to the fact that vacuoles can store K^+ up to 200 mM, a preceding cultivation with adequate K^+ supply can delay the conditions of real substitution (Wakeel et al., 2009). Moreover, the drastic change from K^+ supply to substitution conditions can distort the ionic homeostasis of the plants (Wakeel et al., 2009).

Therefore, in order to determine the potential of Na^+ to replace K^+, the substitution should be started with the beginning of plant cultivation. Studies following this substitution design showed that K^+ can be replaced by Na^+ to a large extent without negative impacts on shoot and root growth (Subbarao et al., 1999, 2003). However, these studies also demonstrated that increased substitution of K^+ by Na^+ inhibited growth of beets, reduced leaf area, and reduced photosynthetic rate (Pi et al., 2014; Subbarao et al., 1999). Unfortunately, up to now it has not been possible to identify the process which is most sensitively limited by the substitution of K^+ by Na^+. Therefore, the aim of the present study was to identify the process which is most sensitive to the substitution of K^+ by Na^+ and thus limits the extent of substitution in young sugar beet plants. We focused on transpiration, growth, and net protein synthesis:

1 K^+ plays a key role in stomatal regulation. Furthermore, it was shown for *Vicia faba* that Na^+ disrupted the ability of stomatal closure leading to unproductive water losses (Slabu et al., 2009). Hence, it is hypothesized that transpiration is most sensitive to the substitution of K^+ by Na^+.
2 The key functions of K^+ ensure numerous processes which are required for plant growth (Hawkesford et al., 2012). We expect that some of the key functions of K^+ cannot be fulfilled by Na^+ and that a variety of processes can be inhibited resulting in poor plant growth due to the substitution of K^+ by Na^+. Therefore, it is hypothesized that the growth of young sugar beet plants is sensitively inhibited due to the substitution of K^+ by Na^+.
3 The requirement of K^+ in protein synthesis was demonstrated in vivo (Besford, 1975) and in vitro (Smith et al., 1982). We assume that the substitution of K^+ by Na^+ could induce K^+ deficiency. Hence, it is hypothesized that protein synthesis is most sensitive to the substitution of K^+ by Na^+.

2. Materials and methods

2.1. Experiment I

2.1.1. Plant material and cultivation

Sugar beet seeds (*Beta vulgaris* L. cv. Felicita), obtained from KWS (Germany), were sown in quartz sand in a growth chamber. The standardized environmental conditions in the climate chamber were 22 °C (16 h)/20 °C (8 h) and 70% relative humidity. The seeds were watered with 1 mM $CaSO_4$ solution which contained 20 µM boric acid. Germination took place in the dark. After germination, the light intensity was increased stepwise until after 4 d a photosynthetic photon flux density (PPFD) of 556 $\mu mol\ m^{-2}\ s^{-1}$ (FieldScout® Quantum Light Meter 3415FSE, Spectrum Technologies, USA) was reached. Eight days after sowing (DAS), the seedlings were transferred into pots (2.8 L) with ¼ strength nutrient solution. With the transfer of the plants into the nutrient solution five treatments were established. The five treatments differed in the extent of substitution of K^+ by Na^+ (Fig. 2). There were four plants in each pot and four replicates of pots for each treatment. The nutrient solution was aerated continuously. Nine DAS, the concentration of the nutrient solution was increased to ½ strength. Eleven DAS, plants were supplied with full-strength nutrient solution. The full-strength nutrient solution contained: 2.5 mM $Ca(NO_3)_2$, 0.3 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 10 µM Fe-EDTA (sodium salt), 20 µM H_3BO_3, 0.5 µM $MnSO_4$, 0.1 µM $ZnSO_4$, 0.2 µM $CuSO_4$, and 0.01 µM $(NH_4)_6Mo_7O_{24}$. The treatments contained (at the level of full-strength nutrient solution) 3.99 mM K^+/0.01 mM Na^+ (for the 0.25% substitution), 3.00 mM K^+/1.00 mM Na^+(for the 25.00% substitution), 2.00 mM K^+/2.00 mM Na^+ (for the 50.00% substitution), 1.00 mM K^+/3.00 mM Na^+ (for the 75.00% substitution), and 0.01 mM K^+/3.99 mM Na^+ (for the 99.75% substitution). K^+ and Na^+ were supplied as KCl and NaCl. After reaching full-strength concentration, the nutrient solution was changed every 3 d. Twenty DAS, the roots were dipped into 0.03% (w/v) Benomyl (DuPont, USA) suspension for 1 min to prevent an infection of the plants by fungi.

2.1.2. Transpiration rate

The loss of water within 48 h was determined by weighing the pots (without plants and lids) 23 and 25 DAS. The transpiration rate was calculated as the loss of water per leaf area during this time period.

2.1.3. N-tester values and harvest of the plants

Twenty-five DAS, 30 random point measurements on the first leaf pairs of plants of each pot were made by means of the handheld Yara N-Tester® (Yara, Norway). This non-destructive method allowed an estimation of the chlorophyll concentration of the leaves. After the determination of N-tester values the plants were harvested. Shoots and roots were separated by cutting at the transition of the hypocotyl to the primary root of the young beet. The fresh mass of shoots and roots was recorded. The shoots of three plants of each pot and the roots of all four plants of each pot were dried at 80 °C for 48 h. After drying the roots and shoots, the dry mass was recorded and the plant material was ground. The shoot of the fourth plant of each pot was frozen in liquid nitrogen and stored at −80 °C until analysis.

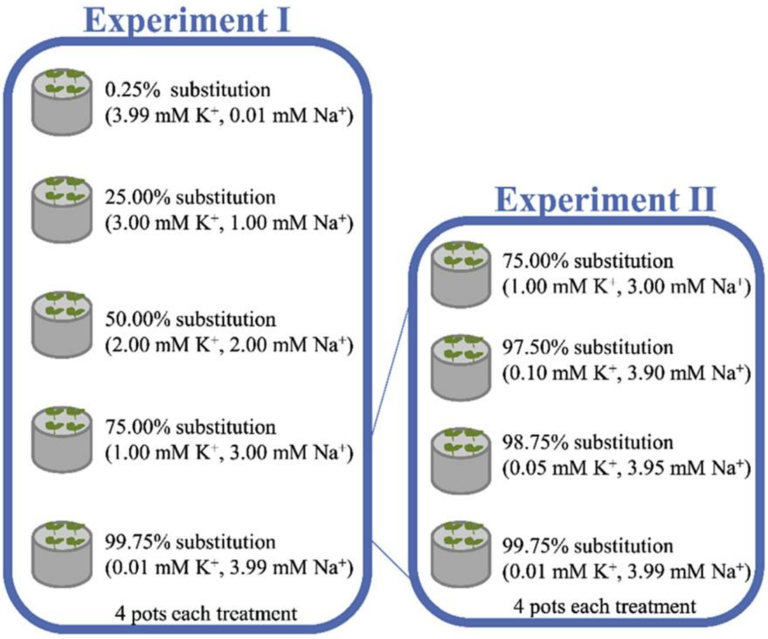

Fig. 2. Experimental design of plant cultivation of sugar beet grown under different substitution of K^+ by Na^+ in the nutrient solution. Plant cultivation was conducted independently in two experiments. In Experiment II, the substitution was further differentiated.

2.1.4. *Leaf area*

Leaf area was determined with a scanner (HP ScanJet7400 C, USA) and the area of leaves was determined by means of the ImageJ 1.48v software (National Institutes of Health, USA).

2.1.5. *Soluble protein*

Ice-cold PBST-Buffer (2.68 mM KCl, 1.47 mM KH_2PO_4, 8.10 mM Na_2HPO_4; pH 7.4; 0.05% (v/v) Tween 20) was added to an aliquot of the ground, frozen shoot material. Extraction of the soluble protein fraction was facilitated by strong vortexing. After incubation on ice for 10 min, the suspension was centrifuged (5 min, 4 °C, 4000g). Centrifugation was repeated until the supernatant was free of visible particles. The protein concentration in the supernatant was determined according to the Bradford method (Bradford, 1976). Bovine serum albumin was used as a reference.

2.1.6. *Cations*

An aliquot of the ground material was dry-ashed at 550 °C overnight. The concentrations of K^+, Na^+, Mg^{2+}, and Ca^{2+} of the shoots and roots were determined using atomic absorption spectrometry (Varian SpectrAA 220 FS, Varian, Australia).

2.1.7. *Sugars*

The dried and ground plant material was extracted in 60 °C water for 30 min. The concentrations of glucose, fructose, and sucrose in the extracts were determined enzymatically (R-Biopharm, Germany).

2.1.8. *Free amino acids*

The concentration of free amino acids was determined according to Rosen (1957) in the water extract. Leucine was used as a reference in this ninhydrin-based photometric method. The concentration of free amino acids was expressed as mmol leucine equivalent per gram dry matter (DM).

2.1.9. *Chloride*

The concentration of chloride (Cl^-) of the shoots and roots was determined potentiometrically in the water extract by means of titration (Aminco Chloride Titrator, USA).

2.2. *Experiment II*

Sugar beet plants (*Beta vulgaris* L. cv. Felicita) were grown according to the protocol described for Experiment I. This experiment was conducted independently in a second climate chamber with the same environmental settings chosen for Experiment I except that the provided PPFD of 365 $\mu mol\ m^{-2}\ s^{-1}$ (FieldScout® Quantum Light Meter 3415FSE, Spectrum Technologies, USA) was lower than in the first climate chamber. In order to prevent a fungal infection, the seeds were coated with Aatiram 65 (Cheminova, Germany) according to the instructions of the manufacturer on the day before sowing. The roots were not dipped into Benomyl solution. In this experiment, four treatments were established (Fig. 2). The treatments contained (at the level of full-strength nutrient solution) 1.00 mM K^+/3.00 mM Na^+ (for the 75.00% substitution), 0.10 mM K^+/3.90 mM Na^+(for the 97.50% substitution), 0.05 mM K^+/3.95 mM Na^+ (for the 98.75% substitution), and 0.01 mM K^+/3.99 mM Na^+(for the 99.75% substitution). The same analyses were conducted as described for Experiment I.

Additionally, the concentration of each proteinogenic free amino acid was determined in the water extract by means of ion-exchange chromatography. Analyses were performed by Frank Gutjahr Chromatografie (Balingen, Germany). The water extract was concentrated with a speed rotary evaporator (4 h, 36 °C, 2 mbar) and the residues were diluted in lithium-acetate buffer (pH 2.2). Twenty µL of clear sample solution were injected into the analyzer. Amino acids were separated using a polymeric cation-exchange column (particle size 4 µm, length 125 mm, diameter 4 mm) with post-column ninhydrin detection (Amino Acid Analyzer LC 3000, Eppendorf-Biotronik, Maintal, Germany). Elution was facilitated using a stepwise pH gradient (lithium-acetate buffer, pH 2.95, 3.33, 4.25, 7.85, 10.60).

2.3. *Experiment III*

Sugar beet plants (*Beta vulgaris* L. cv. Felicita) were grown according to the protocol described for Experiment II. Plants were cultivated with high K^+ and low Na^+ supply (0.25% substitution, 3.99 mM K^+, 0.01 mM Na^+) with different Mg^{2+} concentrations (500 μM Mg^{2+}, 250 μM Mg^{2+}, 100 μM Mg^{2+}, 50 μM Mg^{2+}, and 5 μM Mg^{2+}). By adding 500 μM $CaSO_4$ to all treatments sufficient supply of plants with sulfate was ensured. The same analyses were conducted as described for Experiment I.

2.4. *Statistical analyses*

Results are expressed as means ± standard errors (SE). Means were calculated from four biological replicates. One-way ANOVA with post-hoc analysis was carried out by means of SciStatCalc (http://scistatcalc.blogspot.co.uk/2013/11/home.html). Significant differences ($P = 5\%$) are indicated by different letters.

3. Results

Sugar beet plants were cultivated at various substitution levels (Fig. 2). In order to prevent chloride or osmotic effects the sum of given K^+ and Na^+ concentrations in the nutrient solution was fixed to 4 mM in all treatments.

In Experiment I, the extent of substitution of K^+ by Na^+ was varied from low (0.25% substitution) to almost complete substitution (99.75% substitution) (Fig. 2). In this experiment, the substitution level of 0.25% was considered as control; this treatment contained an ideal concentration of K^+ (3.99 mM) and only traces of Na^+ (0.01 mM). The adequate supply of K^+ in control plants guaranteed the absence of K^+ deficiency. Consequences of K^+ deficiency would superimpose the effects of the substitution of K^+ by Na^+. The K^+ concentration of shoots of control plants was 95 mg g^{-1} DM (Fig. 3 A), clearly above the upper critical K^+ concentration of 60 mg g^{-1} DM for shoots of sugar beet proposed by Bergmann (1992).

As expected, the K^+ and Na^+ concentrations of shoots were changed inversely with the extent of substitution (Fig. 3 A, C). Up to a substitution level of 75.00%, the K^+ concentrations of the shoots were above the lower critical K^+ concentration of 35 mg g^{-1} DM reported by Bergmann (1992) (Fig. 3 A) and the plants did not show any K^+ deficiency symptoms. However, almost total substitution of K^+ (99.75%) caused the K^+ concentration of the shoots to drop significantly below the lower critical concentration (Bergmann, 1992) (Fig. 3 A) and clear symptoms of K^+ deficiency and/or Na^+ toxicity were visible. Significant increases of the Na^+ concentration of the shoots were observed with each further extent of substitution (Fig. 3 C). This increase in Na^+ concentrations fully compensated the decrease in K^+ concentrations up to a substitution level of 50.00% (Fig. 3 E). For the substitution levels of 75.00% and 99.75%, a slight but significant decrease in the sum of K^+ and Na^+ concentration was observed (Fig. 3 E).

The concentration of Ca^{2+} was not negatively affected by the substitution of K^+ by Na^+ except at the highest level of substitution (Fig. 3 G). Substitution of 99.75% of K^+ by Na^+ caused a significant decrease in the Ca^{2+} concentration of the shoots. The lower critical value for the Ca^{2+} concentration of shoots of sugar beet proposed by Bergmann (1992) lies at 7 mg g^{-1} DM. The Ca^{2+} concentration of shoots of plants cultivated in nutrient solution with 99.75% substitution was slightly below this threshold (Fig. 3 G). However, the young leaves of all plants looked healthy and did not show Ca^{2+} deficiency symptoms.

Similar to the Ca^{2+} concentrations, the Mg^{2+} concentrations of shoots were not negatively affected by the substitution of K^+ by Na^+ except at the highest level of substitution of 99.75% (Fig. 3) I. The Mg^{2+} concentration of this treatment was between the upper critical (7 mg g^{-1} DM) and lower critical (3 mg g^{-1} DM) value proposed by Bergmann (1992). No symptoms of Mg^{2+} deficiency were observed.

The aim of present study was to investigate the effects of substitution of K^+ by Na^+ on the selected parameters. Therefore, a possible influence of low Mg^{2+} status must be excluded. Plants were cultivated with high K^+ and low Na^+ supply (3.99 mM K^+, 0.01 mM Na^+) with various Mg^{2+} concentrations (500 μM Mg^{2+}, 250 μM Mg^{2+}, 100 μM Mg^{2+}, 50 μM Mg^{2+}, and 5 μM Mg^{2+}) in the nutrient solution to test which Mg^{2+} concentration of the shoot was necessary to provoke inhibition of the selected parameters. As expected, the Mg^{2+} concentration of shoots significantly decreased with the reducing supply of Mg^{2+} in the nutrient concentration (Fig. 4 A). The parameters shoot dry mass (Fig. 4 B), N-tester values (Fig. 4 D), sugar concentrations of shoots (Fig. 4 E), and soluble protein content of shoots (Fig. 4 F) were not affected by the stepwise reduction of the Mg^{2+} supply to 50 μM. For 50 μM Mg^{2+}-treated plants, a concentration of 1.8 mg g^{-1} DM Mg^{2+} was found in the shoots (Fig. 4 A). The concentrations of free amino acids were not affected by the reduction of the Mg^{2+} supply, even when only 5 μM of Mg^{2+} were supplied in the nutrient solution (Fig. 4 G), which corresponded with a shoot Mg^{2+} concentration of 0.4 mg g^{-1} DM (Fig. 4 A). These concentrations were significantly lower than the Mg^{2+} concentrations found in Experiment I (Fig. 3 I). Therefore, an influence of the Mg status on all determined parameters (except leaf area) was unlikely for Experiment I. For the parameter leaf area a significant reduction was observed for plants supplied with 250 μM Mg^{2+} (Fig. 4 C). The Mg^{2+} concentration of the shoots was 6.1 mg g^{-1} DM for this treatment (Fig. 4 A). We cannot exclude an influence of the low Mg^{2+} status on the leaf area of plants grown in the presence of nutrient solution with 99.75% substitution of K^+ by Na^+ in Experiment I, because the Mg^{2+} concentration of shoots of the 99.75% substitution level (6.2 mg g^{-1} DM) was in a similar range (Fig. 3 I).

To exclude the influence of Cl^- on the processes caused by the substitution of K^+ by Na^+, the concentrations of Cl^- were determined in roots and shoots. The Cl^- concentrations of roots and shoots were between 0.6 and 1.4 mg g^{-1} DM in Experiment I (not shown). According to Broadley et al. (2012) chlorine toxicity could be expected at Cl^- concentrations of 20–30 mg g^{-1} DM of sugar beet leaves. Therefore, it is very unlikely that Cl^- had inhibitory effects in the concentration range found for shoots and roots in Experiment I.

In Experiment I, the low extent of substitution of only 0.25% ensured absence of effects of K^+ deficiency and/or Na^+ toxicity and was considered as control (Fig. 2). Although there was almost 400 times more K^+ than Na^+ in the nutrient solution, the uptake of K^+ by plants of this treatment was only 79 times higher than the uptake of Na^+ (Table 1). The ratio of uptake of these two cations at the whole-plant level is reflected by the ratio of K^+ concentration to Na^+ concentration of the shoot (Table 1).

Stepwise increasing the substitution up to 75.00% did not cause any effect on the determined parameters shoot dry mass (Fig. 5 A), leaf area (Fig. 5 C), N-tester values (Fig. 6 A), sugar concentrations of shoot (Fig. 6 C), soluble protein content of shoots (Fig. 7 A), concentration of free amino acids of shoots (Fig. 7 C), transpiration rate (Supplemental Fig. 1 A) nor induced symptoms of K^+ deficiency and/or Na^+ toxicity. However, the increase of substitution in the nutrient solution was accompanied by a strong decrease of the ratio of K^+: Na^+ uptake by the plants and of the concentration ratio of the

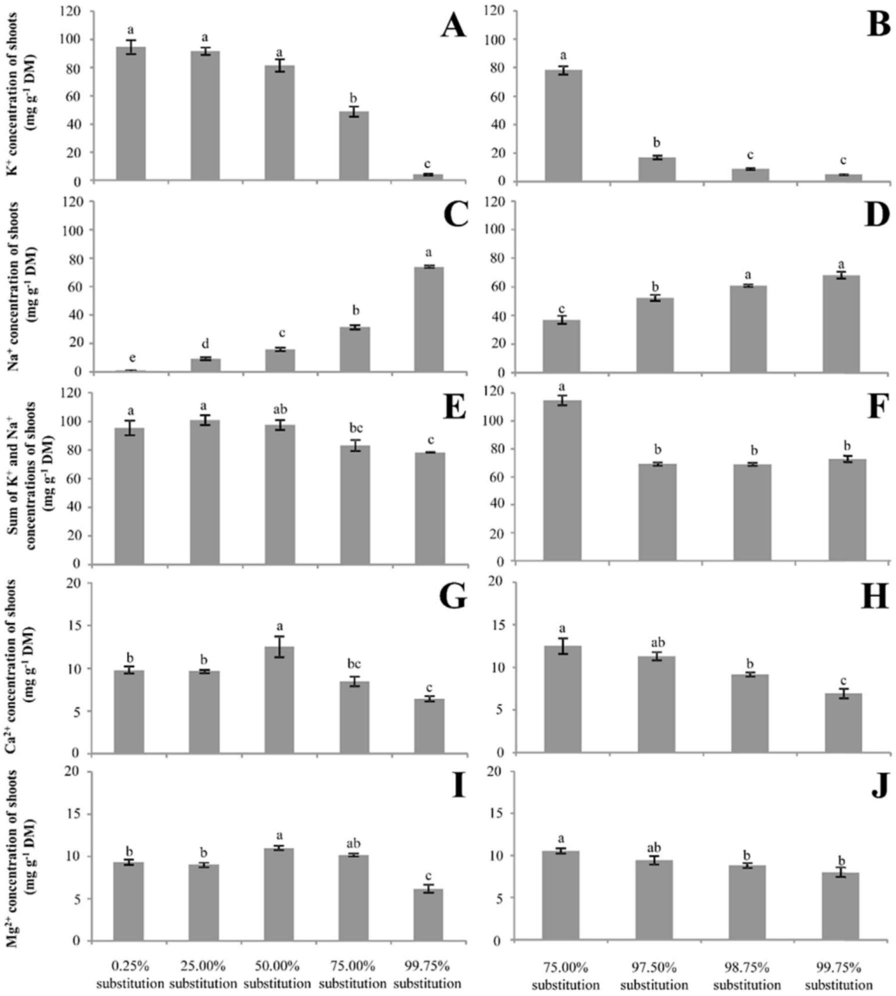

Fig. 3. Effect of different substitution of K^+ by Na^+ in the nutrient solution on the K^+ concentration of shoots in (**A**) Experiment I and (**B**) Experiment II, on the Na^+ concentration of shoots in (**C**) Experiment I and (**D**) Experiment II, on the sum of the K^+ and Na^+ concentration of shoots in (**E**) Experiment I and (**F**) Experiment II, on the Ca^{2+} concentration of shoots in (**G**) Experiment I and (**H**) Experiment II, and on the Mg^{2+} concentration of shoots in (**I**) Experiment I and (**J**) Experiment II. The sum of supplied K^+ and Na^+ concentrations was kept constant at 4 mM: 0.25% substitution (3.99 mM K^+, 0.01 mM Na^+), 25.00% substitution (3.00 mM K^+, 1.00 mM Na^+), 50.00% substitution (2.00 mM K^+, 2.00 mM Na^+), 75.00% substitution (1.00 mM K^+, 3.00 mM Na^+), 97.50% substitution (0.10 mM K^+, 3.90 mM Na^+), 98.75% substitution (0.05 mM K^+, 3.95 mM Na^+), 99.75% substitution (0.01 mM K^+, 3.99 mM Na^+). Results are expressed as means ± SE. Means were calculated from four biological replicates. Significant differences ($P = 5\%$) are indicated by different letters.

shoot (Table 1).

In contrast, 99.75% substitution of K^+ by Na^+ affected plant growth and morphology. The plants showed stunted growth. The first leaf pair of these sugar beet plants showed strong chlorosis. Younger leaves did not exhibit any symptoms. This drastic substitution level inhibited all analyzed parameters (Fig. 5 A, C; Fig. 6 A, C; Fig. 7 A, C) except the transpiration rate (Supplemental Fig. 1 A).

In order to specify the sensitivity level of the affected processes, Experiment II was designed (Fig. 2). Therefore, two further degrees of substitution (97.50% and 98.75%) were included between the level of 75.00% substitution of K^+ by Na^+ in the nutrient solution, which did not cause any effect on the analyzed processes, and the level of 99.75% substitution which inhibited all processes except transpiration in Experiment I.

As in Experiment I, the K^+ concentrations of the shoot decreased (Fig. 3 B), whereas the Na^+ concentrations of the shoot increased with the extent of substitution (Fig. 3 D). The sum of both ion concentrations was similar for the substitution levels of 97.50%, 98.75%, and 99.75%, but significantly lower than the sum of K^+ and Na^+ concentrations of shoots of plants of the 75.00% substitution level (Fig. 3 F). The Ca^{2+} concentrations of shoots of plants were decreased with the increase of the extent of the substitution of K^+ by Na^+ (Fig. 3 H). However, only the Ca^{2+} concentration of shoots of the 99.75% substitution level fell slightly below the lower critical concentration for Ca^{2+} defined by Bergmann (1992) (Fig. 3 H). As in Experiment I, no symptoms of Ca^{2+} deficiency were observed. The Mg^{2+} status of plants of Experiment II was also lowered with the increase of the extent of substitution (Fig. 3 J), but the Mg^{2+} concentrations were significantly above the levels which were necessary to provoke effects on the considered parameters (Fig. 4 A–G).

The plants of the treatment with 75.00% substitution of K^+ by Na^+, which served as control plants in Experiment II, were healthy and did not show any symptoms of K^+ deficiency and/or Na^+ toxicity. The ratio of K^+ uptake to Na^+ uptake and the concentration

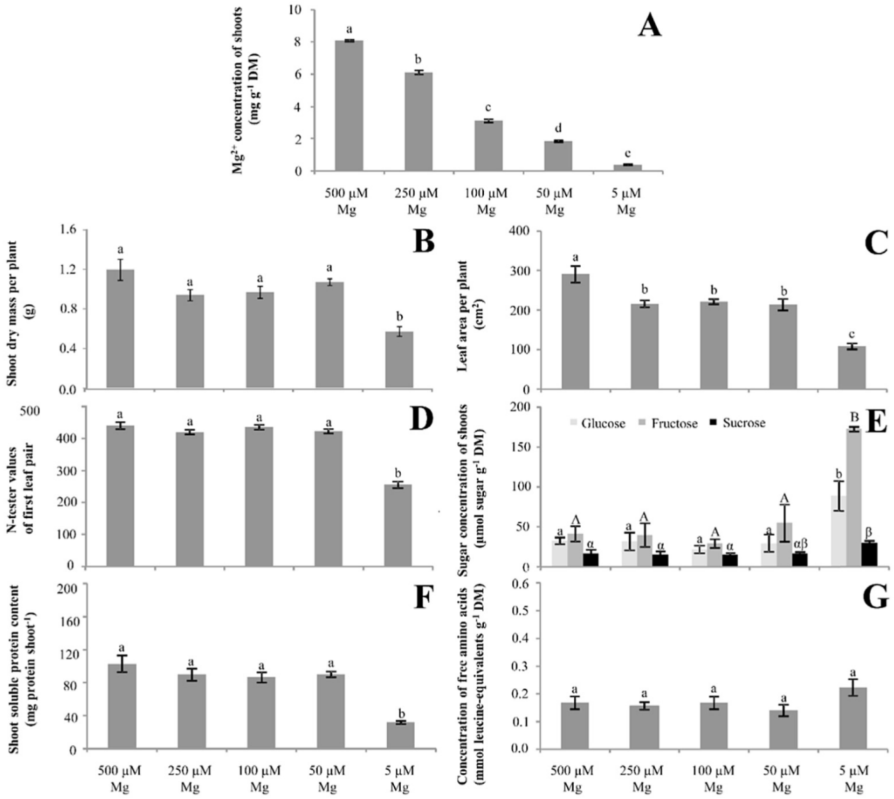

Fig. 4. Effect of various Mg^{2+} concentrations in the nutrient solution (0.25% substitution: 3.99 mM K^+, 0.01 mM Na^+) on the Mg^{2+} concentration of shoots (**A**), on the shoot dry mass (**B**), on the leaf area (**C**), on N-tester values of the first leaf pair of plants (**D**), on the glucose, fructose, and sucrose concentrations of shoots (**E**), on the shoot soluble protein content (**F**), and on the concentration of free amino acids of shoots (**G**). Results are expressed as means ± SE. Means were calculated from four biological replicates. Significant differences ($P = 5\%$) are indicated by different letters.

Table 1
Effect of different substitution of K^+ by Na^+ in the nutrient solution on the ratio of K^+ uptake to Na^+ uptake and on the ratio of K^+ concentration to Na^+ concentration of the shoot. Results are expressed as means ± SE. Means were calculated from four biological replicates.

Extent of substitution of K^+ by Na^+ in the nutrient solution	Concentration of K^+ and Na^+ of the full-strength nutrient solution	Experiment I		Experiment II	
		Ratio of K^+ uptake to Na^+ uptake	Ratio of K^+ concentration to Na^+ concentration of the shoot	Ratio of K^+ uptake to Na^+ uptake	Ratio of K^+ concentration to Na^+ concentration of the shoot
0.25%	3.99 mM K^+ 0.01 mM Na^+	79.31 ± 14.76	74.05 ± 14.84	–	–
25.00%	3.00 mM K^+ 1.00 mM Na^+	6.63 ± 0.64	6.05 ± 0.65	–	–
50.00%	2.00 mM K^+ 2.00 mM Na^+	3.13 ± 0.42	3.11 ± 0.40	–	–
75.00%	1.00 mM K^+ 3.00 mM Na^+	0.93 ± 0.08	0.92 ± 0.07	1.38 ± 0.13	1.26 ± 0.12
97.50%	0.10 mM K^+ 3.90 mM Na^+	–	–	0.21 ± 0.02	0.19 ± 0.02
98.75%	0.05 mM K^+ 3.95 mM Na^+	–	–	0.09 ± 0.01	0.08 ± 0.01
99.75%	0.01 mM K^+ 3.99 mM Na^+	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.00	0.04 ± 0.00

ratio of both ions in the shoot reflected the data found in Experiment I (Table 1). The additional levels (97.50% and 98.75%) of substitution of K^+ by Na^+ allowed the differentiation of sensitivity of parameters to the substitution: The leaf areas (Fig. 5 D), the N-tester values (Fig. 6 B), the sugar concentrations (Fig. 6 D), and the transpiration rates (Supplemental Fig. 1 B) were insensitive to increasing the substitution level from 75.00% to 97.50%. In contrast, the content of soluble protein in the shoot was significantly reduced by the increased level of substitution (Fig. 7 B). There was no significant inhibition of shoot growth when 97.50% of K^+ were replaced by Na^+ in the nutrient solution (Fig. 5 B). Although the contents of soluble proteins were already reduced due to the

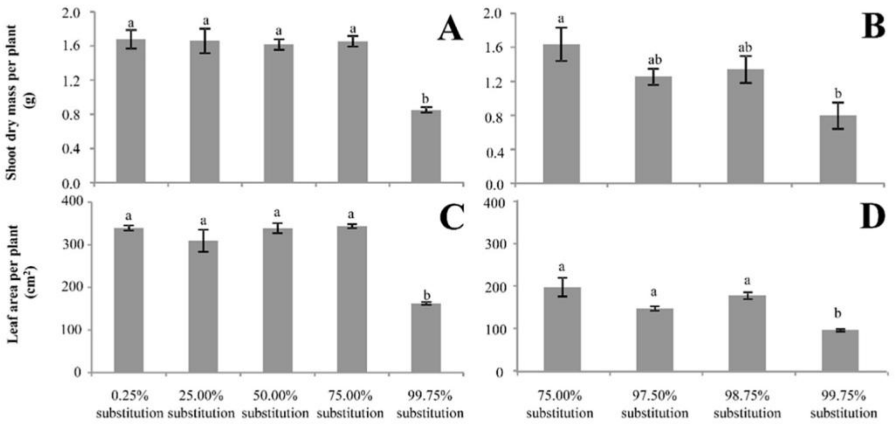

Fig. 5. Effect of different substitution of K^+ by Na^+ in the nutrient solution on the shoot dry mass in (**A**) Experiment I and (**B**) Experiment II, and on the leaf area in (**C**) Experiment I and (**D**) Experiment II. The sum of supplied K^+ and Na^+ concentrations was kept constant at 4 mM: 0.25% substitution (3.99 mM K^+, 0.01 mM Na^+), 25.00% substitution (3.00 mM K^+, 1.00 mM Na^+), 50.00% substitution (2.00 mM K^+, 2.00 mM Na^+), 75.00% substitution (1.00 mM K^+, 3.00 mM Na^+), 97.50% substitution (0.10 mM K^+, 3.90 mM Na^+), 98.75% substitution (0.05 mM K^+, 3.95 mM Na^+), 99.75% substitution (0.01 mM K^+, 3.99 mM Na^+). Results are expressed as means ± SE. Means were calculated from four biological replicates. Significant differences (P = 5%) are indicated by different letters.

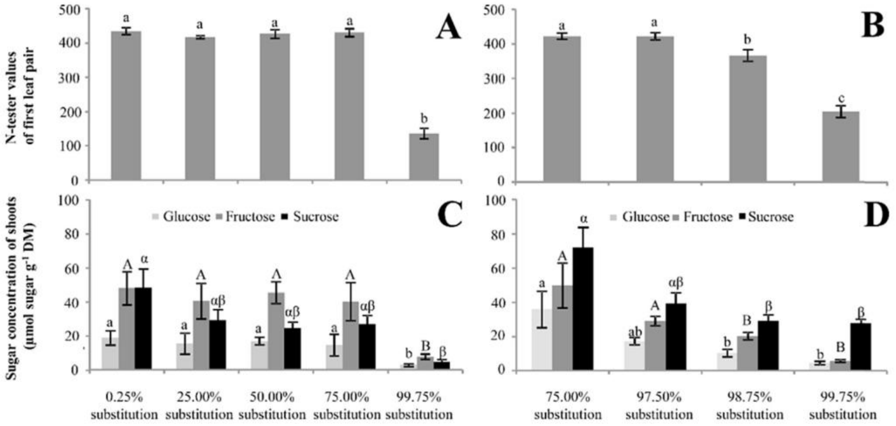

Fig. 6. Effect of different substitution of K^+ by Na^+ in the nutrient solution on the N-tester values of the first leaf pairs of plants in (**A**) Experiment I and (**B**) Experiment II, and on the glucose, fructose, and sucrose concentrations of shoots in (**C**) Experiment I and (**D**) Experiment II. The sum of supplied K^+ and Na^+ concentrations was kept constant at 4 mM: 0.25% substitution (3.99 mM K^+, 0.01 mM Na^+), 25.00% substitution (3.00 mM K^+, 1.00 mM Na^+), 50.00% substitution (2.00 mM K^+, 2.00 mM Na^+), 75.00% substitution (1.00 mM K^+, 3.00 mM Na^+), 97.50% substitution (0.10 mM K^+, 3.90 mM Na^+), 98.75% substitution (0.05 mM K^+, 3.95 mM Na^+), 99.75% substitution (0.01 mM K^+, 3.99 mM Na^+). Results are expressed as means ± SE. Means were calculated from four biological replicates. Significant differences (P = 5%) are indicated by different letters.

substitution level of 97.50%, the plants of this treatment showed no stress symptoms.

The further increase of the substitution to 98.75% caused significant reduction of the N-tester values (Fig. 6 B) and the sugar concentrations (Fig. 6 D), besides the already reduced protein contents (Fig. 7 B). A significant increase in the concentrations of free amino acids was observed when 98.75% of K^+ were substituted by Na^+ (Fig. 7 D). The transpiration rate was not affected at a substitution level of 98.75% (Supplemental Fig. 1 B). Also, the parameters shoot growth and leaf area were not further reduced by the increase of the substitution level to 98.75%; instead a slight but not significant recovery of both parameters was observed (Fig. 5 B, D). The plants did not show any symptoms of K^+ deficiency or Na^+ toxicity.

In line with Experiment I, plants with 99.75% substitution of K^+ by Na^+ showed symptoms of K^+ deficiency or Na^+ toxicity and all parameters (Fig. 5 B, D; Fig. 6 B, D; Fig. 7 B) except the transpiration rates (Supplemental Fig. 1 B) were inhibited. The concentrations of free amino acids were strongly increased due to this almost complete substitution of K^+ by Na^+ (Fig. 7 D; Table 2). The ratio of K^+ uptake to Na^+ uptake and the concentration ratio of both ions of shoots of this substitution level were very similar to the values of Experiment I (Table 1).

4. Discussion

4.1. The transpiration was not affected by the substitution of K^+ by Na^+

It was shown that Na^+ inhibited stomatal closure of faba bean leading to uncontrolled water losses (Slabu et al., 2009), hence, it was expected that transpiration would be affected by the substitution of K^+ by Na^+. Surprisingly, the transpiration rates of young sugar beet plants were not affected by the substitution of K^+ by Na^+

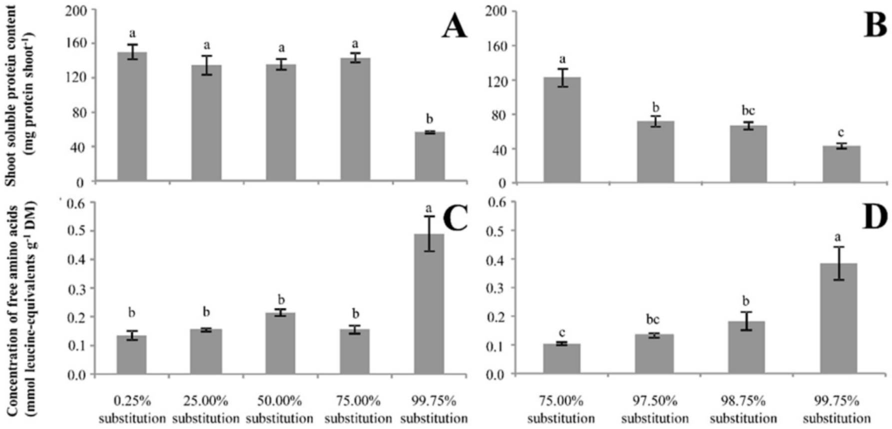

Fig. 7. Effect of different substitution of K^+ by Na^+ in the nutrient solution on the shoot soluble protein content in (**A**) Experiment I and (**B**) Experiment II, and on the concentration of free amino acids of shoots in (**C**) Experiment I and (**D**) Experiment II. The sum of supplied K^+ and Na^+ concentrations was kept constant at 4 mM: 0.25% substitution (3.99 mM K^+, 0.01 mM Na^+), 25.00% substitution (3.00 mM K^+, 1.00 mM Na^+), 50.00% substitution (2.00 mM K^+, 2.00 mM Na^+), 75.00% substitution (1.00 mM K^+, 3.00 mM Na^+), 97.50% substitution (0.10 mM K^+, 3.90 mM Na^+), 98.75% substitution (0.05 mM K^+, 3.95 mM Na^+), 99.75% substitution (0.01 mM K^+, 3.99 mM Na^+). Results are expressed as means ± SE. Means were calculated from four biological replicates. Significant differences ($P = 5\%$) are indicated by different letters.

Table 2
Effect of different substitution of K^+ by Na^+ in the nutrient solution on the concentrations of free amino acids of shoots in Experiment II. Results are expressed as means ± SE. Means were calculated from four biological replicates. Significant differences ($P = 5\%$) are indicated by different letters.

Amino acid	75.00% substitution (1.00 mM K^+, 3.00 mM Na^+)	97.50% substitution (0.10 mM K^+, 3.90 mM Na^+)	98.75% substitution (0.05 mM K^+, 3.95 mM Na^+)	99.75% substitution (0.01 mM K^+, 3.99 mM Na^+)
	Concentration (mmol g^{-1} DM)	Concentration (mmol g^{-1} DM)	Concentration (mmol g^{-1} DM)	Concentration (mmol g^{-1} DM)
Alanine	24.5 ± 2.5 a	32.0 ± 2.7 ab	50.9 ± 9.4 bc	64.3 ± 7.0 c
Arginine	1.7 ± 0.6 a	1.6 ± 0.4 a	3.2 ± 0.5 a	10.8 ± 2.8 b
Aspartate	2.1 ± 0.2 a	3.0 ± 0.3 a	3.9 ± 0.3 a	8.9 ± 1.3 b
Cysteine	0.7 ± 0.0 a	0.7 ± 0.0 a	0.9 ± 0.1 ab	1.0 ± 0.1 b
Glycine	1.9 ± 0.3 a	3.1 ± 0.3 a	6.1 ± 1.7 a	10.8 ± 1.5 b
Glutamate	3.6 ± 0.0 a	3.9 ± 0.9 a	12.5 ± 5.6 ab	31.3 ± 9.4 b
Histidine	0.2 ± 0.0 a	0.3 ± 0.0 a	0.5 ± 0.1 a	1.7 ± 0.6 b
Isoleucine	1.5 ± 0.2 a	1.8 ± 0.3 a	4.3 ± 1.4 a	15.5 ± 3.0 b
Leucine	1.6 ± 0.2 a	1.8 ± 0.2 a	2.9 ± 0.4 a	6.3 ± 0.6 b
Lysine	0.5 ± 0.1 a	0.6 ± 0.1 a	0.9 ± 0.1 ab	1.2 ± 0.2 b
Methionine	Not detectable	Not detectable	Not detectable	Not detectable
Phenylalanine	0.5 ± 0.1 a	0.6 ± 0.1 a	1.0 ± 0.2 a	2.9 ± 0.4 b
Proline	1.7 ± 0.1 a	2.0 ± 0.1 a	3.1 ± 0.4 a	6.5 ± 1.6 b
Serine	2.8 ± 0.5 a	3.5 ± 0.4 a	5.8 ± 1.6 a	10.9 ± 1.7 b
Threonine	1.3 ± 0.2 a	1.6 ± 0.2 a	2.3 ± 0.4 a	4.0 ± 0.6 b
Tyrosine	0.9 ± 0.2 a	1.1 ± 0.2 a	2.6 ± 1.0 a	6.8 ± 2.3 b
Valine	1.9 ± 0.3 a	2.8 ± 0.4 a	6.2 ± 1.9 a	26.8 ± 6.5 b

in the nutrient solution, even at the substitution level of 99.75%. This is in accordance with Subbarao et al. (2003). They showed that stomatal conductance was not affected in the red beet genotype Ruby Queen when 98% of K^+ were substituted by Na^+ in the nutrient solution.

It is assumed that stomatal opening and closing was not affected by the substitution because the transpiration rate was not affected by the substitution. It cannot be excluded that the K^+ concentration of stomatal guard cells was still high enough to enable efficient stomatal regulation, even under the conditions of almost complete substitution of K^+ by Na^+. This could be possible as a consequence of high mobility of K^+ within the plants (Marschner et al., 1981), efficient compartmentalization of Na^+ (Xia et al., 2002), and selective exclusion of Na^+ from guard cells (Schroeder, 1988). The mobility of K^+ in the sugar beet plants was apparent in the plants of the highest substitution level which showed K^+-deficiency symptoms (or Na^+-toxicity) in the older leaf pairs, but not in the young leaves.

However, it is possible that in contrast to faba bean K^+ can indeed be replaced by Na^+ for efficient stomatal regulation in sugar beet. One prerequisite for at least partial replacement of K^+ by Na^+ in this function is a high membrane permeability of Na^+. Possible candidates of channels/transporters in plasma membrane and tonoplast of guard cells allowing Na^+ fluxes were suggested by Kronzucker et al. (2013). However, as emphasized by the authors, a specific demonstration that these channels/transporters ensure the required membrane permeability of Na^+ for efficient opening and closing of guard cells is still missing. In conclusion, stomatal regulation was an extremely insensitive process when K^+ was substituted by Na^+ in sugar beet.

4.2. The effect of substitution of K^+ by Na^+ on growth

Due to the high number of specific functions of K^+ in plant growth, it was expected that Na^+ cannot fulfill all key functions of K^+ and thus several processes would be inhibited in parallel due to the substitution of K^+ by Na^+. Therefore, it was hypothesized that the growth of young sugar beet plants is inhibited due to the substitution of K^+ by Na^+.

Plant growth may be described as the increase in biomass. The parameters shoot and root dry mass on the day of harvest were chosen in order to describe the effects of the substitution of K^+ by Na^+ on plant growth. Shoot dry mass was significantly inhibited when 99.75% of the K^+ supply were substituted by Na^+ (Fig. 5 A, B). This is in line with the substitution experiments of Pi et al. (2014) and Subbarao et al. (1999). They reported growth reductions in *Beta vulgaris* by the substitution levels of 99% and 95%, respectively.

In the present study, no stimulation of growth was observed due to the substitution of K^+ by Na^+. In contrast, a stimulation of growth was reported in some substitution studies. It was shown that the extent of growth stimulation was different for the tested genotypes (Subbarao et al., 1999). Marschner et al. (1981) suggested that the differences in the response to the substitution could be explained with differences in the distribution pattern of K^+ and Na^+ at the tissue and cellular level and the existence of isoenzymes which differ in their dependency on K^+. Root growth was more sensitive to the substitution of Na^+ by K^+ than the growth of shoots (Supplemental Fig. 2 A, B; Fig. 5 A, B). This was also found by Marschner et al. (1981) and Subbarao et al. (1999).

The parameter leaf area was used to describe the effects of the substitution of K^+ by Na^+ on extension growth. In both substitution experiments of our study the substitution level of 99.75% decreased the leaf area by about 50% (Fig. 5 C, D). This is in line with findings of Subbarao et al. (1999). According to Lockhart's equation, extension growth is determined by effective turgor pressure and cell-wall extensibility (Lockhart, 1965). Reduction of the turgor pressure and/or cell-wall extensibility could be responsible for the observed inhibition of extension growth. The effect of the substitution of K^+ by Na^+ on turgor was not measured in this study. However, the fact that relative water contents of shoots and roots (calculated as g water g^{-1} DM) were not changed by any level of substitution (not shown) indicates that turgor was not affected by the substitution. According to the acid-growth theory the PM H^+-ATPase plays a key role in extension growth. Acidification of the apoplast by PM H^+-ATPase activity enables activation of pH-dependent enzymes which mediate cell-wall loosening and thereby increase cell-wall extensibility (Hager, 2003). Wakeel et al. (2011) showed that the proton pumping activity of plasma membrane H^+-ATPase of sugar beet was inhibited in vitro when 75% of K^+ was substituted by Na^+ in the reaction buffer. Although it was not possible to determine the K^+ and Na^+ concentration in the cytosol, the K^+: Na^+ concentration ratio at the whole-tissue level for the 99.75% substitution treatment (Table 1) indicates that inhibition of the ATPase activity under such conditions was likely and might have caused the reduction in extension growth.

One prerequisite for growth is the availability of carbohydrates which provide energy and contribute to biomass formation. The concentrations of sugars (glucose, fructose, and sucrose) were chosen as parameters to assess the effect of the substitution on the supply of sugars. The supply of these sugars was already significantly reduced by the substitution of 98.75% of K^+ by Na^+ in the shoots (Fig. 6 D) and roots (not shown). The reduced supply of sugars suggests that photosynthesis was inhibited by the substitution because photosynthates form the basis of sugar synthesis. However, the reduced supply of sugars did not affect growth (Fig. 5 B) at this substitution level.

Also, the decrease in the concentration of the active chlorophyll, which was determined by using the N-tester, indicates that photosynthesis might have been affected by the substitution of K^+ by Na^+. The N-tester values were significantly reduced by the substitution level of 98.75% (Fig. 6 B), which was the same level of substitution associated with reduction of sugar concentrations (Fig. 6 D). This is in accordance with Hampe and Marschner (1982) who also showed that the chlorophyll concentration was reduced by the substitution of 95% K^+ by Na^+ in the nutrition of sugar beet. As in the present study, reduction of chlorophyll concentrations was observed, while growth was not affected by the corresponding level of substitution. Also, the growth of leaf discs of sugar beet on substrate with different concentrations of K^+ and Na^+ indicated that K^+ cannot be substituted by Na^+ in chlorophyll synthesis (Marschner and Possingham, 1975). Besides the reduced chlorophyll concentrations, it is also possible that inhibition of light reaction, dark reaction or the conversion of triosephosphates into hexoses led to the reduced supply of sugars.

Apart from supply of sugars, protein synthesis is a prerequisite for growth. The parameter protein content was significantly reduced by the substitution of 97.50% (Fig. 7 B); however, growth was not affected at this level of substitution (Fig. 5 B). Protein contents were further reduced, when substitution was extended to 99.75% (Fig. 7 B). This further reduction of protein contents, the reduced supply of sugars (Fig. 6 D) and the low Ca^{2+} status (Fig. 3 H) contributed probably to the inhibition of growth during substitution of 99.75% K^+ by Na^+.

It was shown that substitution could be extended to 98.75% without inhibition of growth (Fig. 5 B, D). The supply of sugars and the protein synthesis were already inhibited at a lower extent of substitution compared to the level of substitution required to provoke inhibition of growth. Therefore, it is concluded that growth was sensitive to the substitution of K^+ by Na^+, but growth was not the process which was most sensitive to the substitution.

4.3. Translation is the most sensitive process during substitution of K^+ by Na^+

It was shown by in vivo and in vitro experiments that protein synthesis is highly dependent on K^+ (Besford, 1975; Smith et al., 1982), hence, it was hypothesized that protein synthesis is most sensitive to the substitution of K^+ by Na^+. The soluble protein content, which was chosen as a parameter for net protein synthesis, was already significantly decreased by the substitution of 97.50% of K^+ by Na^+ in the nutrient solution (Fig. 7 B). Growth and the supply of sugars were inhibited at higher levels of substitution (Fig. 5 B, D; Fig. 6 D) and the transpiration (Supplemental Fig. 1 B) was insensitive to the substitution. Therefore, net protein synthesis was most sensitive to the substitution of K^+ by Na^+.

An adequate supply of Mg is an important prerequisite for translation because Mg^{2+} stabilizes the assembly of the ribosomal subunits (Cammarano et al., 1972). Plants of the 97.50% substitution level exhibited a shoot Mg^{2+} concentration of 9.4 mg g^{-1} DM (Fig. 3 J), which was clearly above the critical values defined by Bergmann (1992). Moreover, we showed that shoot Mg^{2+} concentrations of 1.8 mg g^{-1} DM were high enough to ensure protein synthesis (Fig. 4 A, F). Therefore, inhibition of protein synthesis due to a shortage of Mg^{2+} was unlikely during substitution of 97.50% K^+ by Na^+.

There is no evidence that protein synthesis was limited by substrate limitation. The supply of sugars was not affected at this level of substitution (Fig. 6 D). Moreover, an increase in the concentration of free amino acids was observed (Fig. 7 D). The concentration of free amino acids was determined according the method of Rosen (1957). Although this method is very fast and easy, the specificity of this ninhydrin-based assay is limited. Polyamines such as putrescine, spermine, and spermidin are also detected by this method. K^+ deficiency caused not only the accumulation of amino acids in sugar beet (Pi et al., 2014), but also the accumulation of putrescine in plant leaves (e.g. Smith et al., 1982). Therefore, the ninhydrin-based method is not conclusive enough to quantify the concentration of total free amino acids. In order to ensure that the translation was not limited by a shortage of any amino acid, the determination of the concentration of each single proteinogenic free amino acid was necessary. The supply of all proteinogenic amino acids was not affected by the substititution of K^+ by Na^+, the concentrations of the free amino acids even increased with the extent of the substitution (Table 2).

Unfortunately, it was not possible to quantify the concentrations of free methionine, because the concentrations were too low. This is consistent with the results of Pi et al. (2014), who detected only traces of free methionine in the leaves of sugar beet. A limitation of protein synthesis due to a deficiency of methionine, which is part of the aspartate family, is considered as very unlikely, because there was an increase in all other proteinogenic amino acids (Table 2), including all members of the aspartate family. In particular, the accumulation of threonine indicates that the synthesis of methionine can be regarded as ensured up to the formation of homoserine 4–phosphate, because the synthetic pathways of methionine and threonine are identical up to this intermediate (Coruzzi and Last, 2000). A substrate limitation for the formation of cystathionine from homoserine 4–phosphate and cysteine is unlikely due to the accumulation of cysteine (Table 2). The formation of cystathionine is catalyzed by cystathionine γ-synthase (EC 2.5.1.48), the following last two reactions of methionine biosynthesis are catalyzed by cystathionine β-lyase (EC 4.4.1.8) and methionine synthase (EC 2.1.1.13) (Coruzzi and Last, 2000). To our knowledge no dependence on K^+ or inhibition of activity by Na^+ was shown for all three enzymes. Therefore, we assume that biosynthesis of methionine was not affected by the substititution of K^+ by Na^+. That no accumulation of free methionine, in contrast to all other proteinogenic amino acids, was observed due to the substitution of 99.75% could be explained by the fact that a majority of synthesized methionine (about 80%) is converted to *S*-adenosylmethionine (Giovanelli et al., 1985), which is needed for transmethylation reactions in plants and exhibits a feedback inhibition on cystathionine γ-synthase (Onouchi et al., 2004) and, thus, on methionine synthesis.

Protein synthesis was not limited by substrate limitation. Hence, other possible reasons for the inhibition of protein synthesis due to the substitution of K^+ by Na^+ have to be taken into account. It is possible that changes in the cytosolic K^+: Na^+ ratio disturbed transcription or mRNA processing, reduced the activity of aminoacyl-tRNA ligases (EC 6.1.1.1–7, 9–12, 14–24) or impaired the binding of aminoacyl-tRNAs to ribosomes. However, a more likely reason is the inhibition of the peptidyl transferase (peptidyl-tRNA:aminoacyl-tRNA N-peptidyltransferase; EC 2.3.2.12) activity due to changes in the K^+: Na^+ ratio. Peptidyl transferase activity in *E. coli* is activated by K^+ (Maden et al., 1968) and inhibited by Na^+ (Lubin and Ennis, 1964). Nissen et al. (2000) showed that K^+ provides stability to the catalytic center of *Haloarcula marismortui* peptidyl transferase, which is intrinsic to the large ribosomal subunit. K^+ coordinates the orientation of three active residues of the catalytic center. These active residues are also conserved in eukaryotes. This is in line with Flowers and Dalmond (1992), who showed that the substitution of K^+ by Na^+ inhibited the amino acid-incorporating activity of an in vitro wheat germ translation system. We suppose that Na^+ was not able to replace K^+ in its stabilizing function in the catalytic center of peptidyl transferase. This has to be confirmed by further experiments.

5. Conclusions

The extent of substitution of K^+ by Na^+ was varied over a wide range to almost complete substitution. Transpiration was not affected by the substitution. It was shown that substitution could be extended to 98.75% without growth inhibition. Net protein synthesis was most sensitive to the substitution and hence, limited the extent of substitution of K^+ by Na^+ in the nutrition of young sugar beet plants. We suggest that the inhibition of peptidyl transferase activity due to decreased cytosolic K^+: Na^+ concentration ratio is the most sensitive process when K^+ is substituted by Na^+ in sugar beet.

Author contribution

SS designed the study. FF conducted experiments and analyzed data. SS and FF wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.06.009.

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3 Publication 2: *In vitro* protein synthesis of sugar beet (*Beta vulgaris*) and maize (*Zea mays*) is differentially inhibited when potassium is substituted by sodium*

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Research article

In vitro protein synthesis of sugar beet (*Beta vulgaris*) and maize (*Zea mays*) is differentially inhibited when potassium is substituted by sodium

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The substitution of potassium ions (K^+) by sodium ions (Na^+) in the nutrition of plants is restricted. It was shown earlier that net protein synthesis is the process which is most sensitive to the substitution of K^+ by Na^+ in young sugar beet. We hypothesized that the activity of ribosomes is inhibited by the substitution. This hypothesis was tested in an *in vitro* approach. Cytosolic polysomes were isolated from growing leaves of sugar beet and maize by means of differential centrifugation. *In vitro* systems of both plant species were tested for functionality and comparability. Translation was quantified by the ^{35}S-methionine incorporation in TCA-precipitable products. The effect of different substitution levels (0%, 20%, 40%, 60%, and 80% substitution of K^+ by Na^+) on *in vitro* translation was measured. Translation by polysomes of both plant species was significantly inhibited by the substitution. However, the translation by maize polysomes was more negatively affected by the substitution. A significant decrease in the translation by maize polysomes was observed already when 20% of K^+ were replaced by Na^+, whereas in the case of sugar beet, the translation was inhibited firstly at the substitution level of 40%. The *in vitro* results show that the process of translation itself is disturbed by the substitution and indicate a higher tolerance of sugar beet polysomes to increased Na^+ concentrations and Na^+/K^+ ratios compared to polysomes of maize. We propose that this tolerance contributes to the salt resistance of sugar beet.

1. Introduction

Although potassium ions (K^+) and sodium ions (Na^+) show many similarities, the substitution of K^+ by Na^+ in the nutrition of plants is restricted (Benito et al., 2014). Although unspecific osmotic functions of K^+ (such as contribution to turgor) can be taken over by Na^+, Na^+ cannot completely fulfill specific functions of K^+ (such as enzyme activation) in plants (Schubert, 2015). Therefore, potassium is a macronutrient for all higher plants, while sodium is considered as a micronutrient only for some C4 plants and halophytes (Johnston et al., 1989).

Recently we showed that net protein synthesis was the process most sensitive to the substitution of K^+ by Na^+ in young sugar beet and hence limited the extent of the substitution (Faust and Schubert, 2016). However, the accumulation of free amino acids indicated that net protein synthesis was not restricted by substrate limitation.

In the plant cytosol protein synthesis is enabled by 80 S ribosomes, each consisting of a large 60 S subunit and a small 40 S subunit. Both subunits comprise ribosomal RNA and ribosomal proteins (Wilson and Cate, 2012). During translation ribosomes form polysomes, so that multiple ribosomes are engaged in translation on a single mRNA. The process of translation is divided into three phases: initiation, elongation, and termination. Ribosomes are considered as ribozymes, since peptide bond formation during elongation and hydrolysis of peptidyl-tRNA during termination of protein synthesis are catalyzed by ribosomal RNA of peptidyl transferase (EC 2.3.2.12) (Beringer and Rodnina, 2007; Polacek and Mankin, 2005).

Activation of many enzymes by K^+ is based on the electrostatic binding of K^+ to enzymes, which brings about conformational changes allowing stimulation of enzyme activity. The larger diameter of the hydrated Na^+ does not allow conformational changes required for optimal enzyme activity (Schubert, 2015). For prokaryotic and eukaryotic ribosomes from *Escherichia coli* (Conway,

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Abbreviations: cpm, counts per minute; PM, plasma membrane.

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1964; Lubin and Ennis, 1964), *Halobacterium cutirubrum* (Bayley and Griffiths, 1968), rat liver (Sachs, 1957), rabbit reticulocytes (Cahn and Lubin, 1978), and wheat germ (Flowers and Dalmond, 1992; Gibson et al., 1984) it was shown that the substitution of K^+ by Na^+ inhibits *in vitro* protein synthesis. Therefore, it is hypothesized that *in vitro* protein synthesis of cytosolic polysomes from sugar beet is also inhibited due to the substitution of K^+ by Na^+. Inhibition of *in vitro* protein synthesis could explain the observed inhibition of net protein synthesis *in vivo* (Faust and Schubert, 2016).

The substitution of K^+ by Na^+ *in vitro* simulates the cytosolic conditions present in the second phase of salt stress. This phase is characterized by ion toxicity due to the accumulation of Na^+ and/or the induced deficiency of K^+ (Fortmeier and Schubert, 1995; Munns, 1993; Munns and Tester, 2008). Sugar beet and maize strongly differ in their level of resistance in the second phase of salt stress. The sensitivity of maize towards Na^+ is much higher compared to sugar beet. Sugar beet as a descendant of sea beet (*Beta vulgaris* ssp. *maritima*) shows halophytic traits (Greenway and Munns, 1980) and is able to cope with high tissue Na^+ concentration due to the active sequestration of Na^+ into vacuoles (Adler et al., 2010). Successful compartmentalization of Na^+ (and chloride) is considered as the basis of salt resistance of halophytic plants (Greenway and Munns, 1980).

An additional adaptation of the metabolism of halophytes to increased cytosolic Na^+ concentrations has long been considered as unlikely, since several cytosolic enzymes from halophytes exhibited similar *in vitro* sensitivity to high Na^+ concentrations as those of glycophytes (Flowers, 1972; Greenway and Osmond, 1972). However, an adaptation of the protein synthesis of halophytic plants to high cytosolic Na^+ concentrations was considered as possible by Greenway and Munns (1980). *In vitro* results of Flowers and Dalmond (1992) gave first indications that polysomes of halophytes could be more tolerant to low K^+ and high Na^+ concentrations than polysomes of glycophytes. In this study, the K^+ concentration was kept at a very low concentration of 25 mM and the effect of addition of 100 mM Na^+ on ^{35}S-methionine incorporation into protein was tested. The addition of Na^+ led to a strong increase of methionine incorporation by polysomes of the halophytes *Atriplex isatidea*, *Inula crithmoides*, and *Suaeda maritima*, whereas almost no stimulation or inhibition of incorporation was observed for polysomes of the glycophytes *Pisum sativum* and *Triticum aestivum*. However, in order to test whether Na^+ can maintain protein synthesis in halophytes, a substitution of K^+ by Na^+ must be performed at the level of optimal potassium supply. The aim of this work was to test whether the polysomes of sugar beet are more resistant to the stepwise substitution of K^+ (based on an optimal K^+ supply) by Na^+ than polysomes of the glycophyte maize.

2. Materials and methods

2.1. Plant cultivation and harvest of plant material

Sugar beet plants (*Beta vulgaris* L. cv. Felicita) were grown in water culture according to the protocol described in Faust and Schubert (2016). There were four biological replicates.

Maize seeds (*Zea mays* L. cv. Pioneer 3906) were soaked in 1 mM $CaSO_4$ and germinated in sandwich culture. On 7th day the seedlings were transferred to pots containing ¼ strength nutrient solution. There were four plants per 4 L pot and four biological replicates. The plants were transferred into ½ strength nutrient solution and full-strength nutrient solution on 9th and 11th day, respectively. Full-strength nutrient solution (according to Hütsch et al., 2016) contained: 2 mM $Ca(NO_3)_2$, 0.2 mM KH_2PO_4, 1 mM K_2SO_4, 2 mM $CaCl_2$, 0.75 mM $MgSO_4$, 0.2 mM Fe-EDTA, 1 mM Na_2SiO_3, 1 μM H_3BO_3, 2 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.1 μM $NiSO_4$, and 0.01 μM $(NH_4)_6Mo_7O_{24}$. The nutrient solution was renewed every third day. The standardized environmental conditions in the climate chamber were 26 °C (16 h)/18 °C (8 h) and 50% relative humidity.

On 23rd day, the sugar beet and maize plants were harvested. Young growing leaves (the second and third leaf pairs of sugar beet and the sixth and seventh leaves of maize) were separated from remaining shoot parts. The leaves were washed three times with deionized, with double-deionized, and with autoclaved double-deionized water, respectively. Adhering water was removed with paper tissue. The leaves were frozen in liquid nitrogen. The frozen plant material was ground with mortar and pestle to a fine powder and stored at −80 °C until isolation of polysomes.

2.2. Isolation of polysomes

Polysomes were isolated from 60 g leaf material using the protocol of Rivera et al. (2015) with some modifications. The ground plant material was suspended in cold plant extraction buffer (50 mM Tris-HCl (pH 9.0), 30 mM $MgCl_2$, 400 mM KCl, 17% (w/w) sucrose, 0.5 mg/mL heparin and 5 mM DTT). After homogenization four times with an ice-cold Waring blender (15 s pulses in 1 min intervals), the homogenate was filtered through two layers and later through eight layers of autoclaved Miracloth tissue (Merck Millipore) on ice. In order to remove cell debris the suspension was centrifuged at 1076 *g* (4 °C, 7 min, Sorvall AH 629; 36 mL polyallomer tubes). The supernatant was transferred into sterile polypropylene tubes (Sarstedt) and 0.2 volume of 10% Triton X-100 was added. After vortexing, the supernatant was centrifuged at 17,210 *g* (4 °C, 20 min, Sorvall AH 629, 36 mL polyallomer tubes). 5.8 g of plant sucrose cushion buffer (20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 100 mM NH_4Cl, 60% (w/w) sucrose) were given into a 13.5 mL thinwall polypropylene tube (326814, Beckman) and the supernatant (about 7.0 g; the exact weight was adjusted to a total weight of 19.50 g including tube, sucrose cushion, supernatant and aluminum cap) was carefully given on top of the cushion. After centrifugation at 150,643 *g* (4 °C, 8 h, Beckman 50 Ti), the resulting pellet was washed four times at 4 °C with cold 1 mL resuspension buffer (20 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 50 mM KCl, 5 mM DTT). By turning the centrifugation tubes up-side down washing solution was removed from pellets. The pellet of each tube was resuspended in 60 μL of resuspension buffer by carefully pipetting up and down. Resuspended pellets were combined in one of the tubes. Aliquots of 20 μL were quick-frozen in liquid nitrogen and stored at −80 °C. Isolation of polysomes was carried out independently for the four biological replicates of sugar beet and maize on separate days. The buffers were prepared freshly on the day before isolation.

The concentration of polysomes was determined according to Vayda (1995). Since the concentrations varied, the individual concentration of each biological replicate was adjusted with resuspension buffer to 1.4 μg polysomes μL^{-1} before use in the *in vitro* translation assay.

2.3. In vitro translation

In the *in vitro* translation assay 21 μg of polysomes (15 μL) were used. 5 μL of ribosome-depleted wheat-germ extract were included in the assay to ensure the presence of tRNAs, aminoacyl-tRNA synthetases, initiation, elongation, and termination factors. Wheat-germ ribosomes were separated from the commercially available wheat germ extract (Promega) by centrifugation at 175,000 *g* (4 °C, 5 h, Beckman TLA-100.1, 0.5 mL thick-wall polycarbonate tubes) according to Stupina and Simon (2013). The final concentrations in the 50 µL *in vitro* translation reaction volume were: 80 µM of each proteinogenic amino acid (Promega), 25 µCi ^{35}S methionine (>1000 Ci/mmol; stabilized) (Hartmann Analytic), 0.4 µg luciferase control RNA (Promega), 40 U RNasin® Plus RNase Inhibitor (Promega), 5 mM DTT (dissolved in 20 mM HEPES, KOH, pH 7.6), 0.1 mM GTP (dissolved in 20 mM HEPES, KOH, pH 7.6), 1.2 mM ATP (dissolved in 20 mM HEPES, KOH, pH 7.6), 10 mM creatine phosphate (dissolved in 20 mM HEPES, KOH, pH 7.6), 50 µg mL^{-1} creatine phosphokinase from rabbit muscle (dissolved in 50% glycerol), 25 µg mL^{-1} chloramphenicol (pre-solved in ethanol (10 mg mL^{-1}) and diluted in 20 mM HEPES, KOH, pH 7.6). Magnesium (Mg^{2+}), K^+, and Na^+ were given as acetate salts and dissolved in 20 mM HEPES, KOH, pH 7.6. In the context of varying K^+ and Na^+ in the translation assay, the concentrations of these ions in all assay components were considered.

Reaction mixtures were incubated at 25 °C for 90 min. Translation reactions were stopped by transfer of reaction tubes on ice. An aliquot of 5 µL of the reaction assay was given to 95 µL of 1 M NaOH, mixed by pipetting and incubated at 37 °C for 10 min. In order to precipitate translation products, 900 µL of cold 25% (w/v) TCA with 2% (w/v) casein hydrolyzate were added and incubated for 30 min on ice. 250 µL of this mixture were given onto a filter (Whatman®glass microfiber filter, binder-free, grade GF/C) which was pre-wetted with 250 µL of cold 5% (w/v) TCA and placed in a glass microanalysis filter holder (Rocker). The precipitates were collected after vacuum filtration, washed four times with 1 mL cold 5% (w/v) TCA and 1 mL acetone, respectively. In order to determine the total counts present in the reaction, 5 µL of TCA-treated aliquot were given directly onto a filter (filters were not washed with TCA or acetone). The washed and non-washed filters were dried for 1 h at room temperature and transferred into scintillation vials with 3 mL of scintillation cocktail (Ultima Gold F, Perkin Elmer). After incubation of the filters overnight in scintillation cocktail, counts per minute (cpm) were measured with a liquid scintillation counter (LSC 6500, Beckman). For calculating the ^{35}S-methionine incorporation into translation products the following formula was used:

$$^{35}S - methionine\ incorporation\ (in\ percent) = \frac{cpm\ of\ washed\ filter \times 100}{(cpm\ of\ non - washed\ filter) \times 50} \tag{1}$$

2.4. Statistics

Results are expressed as means (±standard errors). Means were calculated from four biological replicates unless otherwise indicated. Two-way ANOVA with post-hoc analysis (Tukey-HSD) was carried out by means of Statistica (Version 12, Tulsa, Oklahoma, USA). Significant differences ($P < 0.05$) are indicated by different letters.

3. Results

3.1. Suitability of the in vitro systems of polysomes isolated from sugar beet and maize

Polysomes were isolated from growing leaves of sugar beet and maize by means of differential centrifugation. Although the resuspended polysomal pellet also contained monosomes and ribosomal subunits, the used fraction is termed 'polysomes' for reasons of simplicity.

The suitability of the *in vitro* translation systems of sugar beet and maize polysomes was tested by stepwise omission of reaction mixture components (Table 1). Both systems showed absolute requirement of polysomes for ^{35}S-methionine incorporation. Both systems responded similarly to the omission of supernatant: The incorporation of ^{35}S-methionine decreased to about one-fifth of the incorporation of complete system. Both tested systems relied on the supply of metabolic energy and the presence of an energy-regenerating system. Amino acid incorporation into translation products was slightly increased in the absence of chloramphenicol (+8% for sugar beet and +18% for maize). Translation of both *in vitro* systems was not inhibited by omitting unlabeled amino acids or exogenous mRNA.

3.2. Identification of optimal Mg^{2+} and K^+ concentrations for in vitro translation by polysomes isolated from sugar beet and maize

Since a sufficient supply of Mg^{2+} and K^+ is required for proper functioning of protein synthesis, it was necessary to identify Mg^{2+} and K^+ concentrations which meet the requirements of cytosolic polysomes of both plant species. Protein synthesis was quantified by incorporation of ^{35}S-methionine into translation products. Since the presence of chloroplastic and mitochondrial ribosomes and polysomes could not be completely excluded, activity of the organellar ribosomes was inhibited by adding chloramphenicol in the *in vitro* assays.

While an optimal Mg^{2+} concentration (in the presence of 151 mM K^+) of 4–6 mM Mg^{2+} was found for protein synthesis with polysomes from maize, protein synthesis of sugar beet polysomes was highest at 8 mM Mg^{2+} (Fig. 1).

Whereas optimal K^+ supply (in the presence of 6 mM Mg^{2+}) was in the range of 101–126 mM K^+ in maize, incorporation of ^{35}S-methionine by polysomes of sugar beet was highest in presence of 151 mM K^+ (Fig. 2).

3.3. Effect of the substitution of K^+ by Na^+ on in vitro translation by polysomes isolated from sugar beet and maize

In order to meet the requirements of the systems from both plant species, Mg^{2+} and K^+ were offered at concentrations of 6 mM and 126 mM, respectively, in the following substitution experiments. Due to presence of Na^+ in the assay components, the minimal Na^+ concentration in the reaction mixture of control and all substitution treatments was 2.7 mM. The K^+ supply of 126 mM served as a control (0% substitution). K^+ was stepwise replaced by Na^+ (20% substitution = 100.8 mM K^+, 25.2 mM Na^+, 40% substitution = 75.6 mM K^+, 50.4 mM Na^+, 60% substitution = 50.4 mM K^+, 75.6 mM Na^+, and 80% substitution = 25.2 mM K^+, 100.8 mM Na^+) (Figs. 3 and 4).

No significant differences were found in the ^{35}S-methionine incorporation of the control treatments (0% substitution) of both plant species (Fig. 3). However, the translation of maize polysomes was significantly lower than the translation of sugar beet polysomes when 20% of K^+ were substituted by Na^+ (Fig. 3). The

Table 1
Effect of reaction mixture composition on the relative *in vitro* incorporation of ^{35}S-methionine into translation products by polysomes isolated from sugar beet and maize. *In vitro* translation assays were prepared as described in Material and methods. Mg^{2+} and K^+ were offered at concentrations of 6 mM and 126 mM, respectively. For the omitted reaction mixture components the equivalent volume of solvent (resuspension buffer; 20 mM HEPES, KOH, pH 7.6; 50% glycerol; RNAse free water) was added to keep the assay volume constant at 50 μL. Data obtained for modified reaction mixtures are expressed relative to the complete reaction mixture. Results are expressed as means of three technical replicates from one biological replicate of both plant species.

Reaction mixture	Sugar beet *in vitro* system ^{35}S-methionine incorporation (%)	Maize *in vitro* system ^{35}S-methionine incorporation (%)
Complete	100	100
Half of polysomes	47	54
Without polysomes	2	3
Without supernatant	22	16
Without GTP, ATP, creatine phosphate, kinase	10	8
Without chloramphenicol	108	118
Without unlabeled amino acids	99	100
Without exogenous RNA	99	102

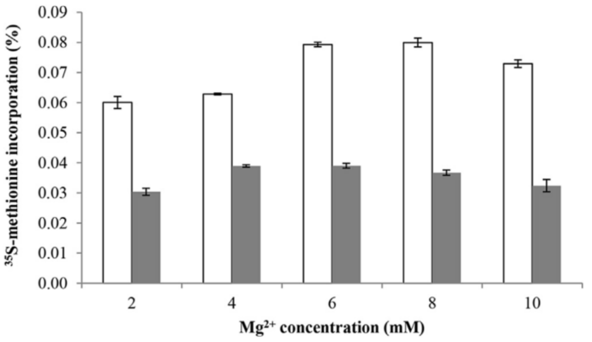

Fig. 1. Effect of various Mg^{2+} concentrations (in mM) on the *in vitro* incorporation of ^{35}S-methionine into translation products by polysomes isolated from sugar beet (white columns) and maize (grey columns). Reactions were performed in the presence of 151 mM K^+. *In vitro* translation assays were prepared as described in material and methods. Data are the means of two biological replicates (±standard errors).

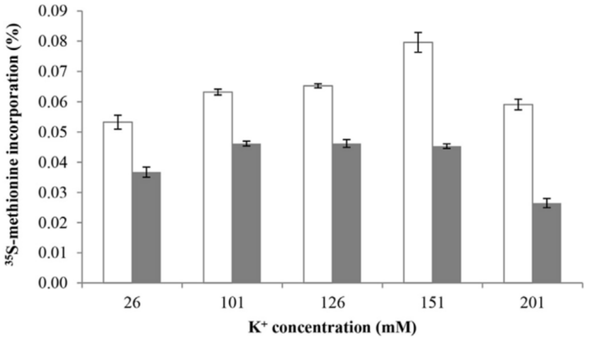

Fig. 2. Effect of various K^+ concentrations (in mM) on the *in vitro* incorporation of ^{35}S-methionine into translation products by polysomes isolated from sugar beet (white columns) and maize (grey columns). Reactions were performed in the presence of 6 mM Mg^{2+}. *In vitro* translation assays were prepared as described in Material and methods. Data are the means of two biological replicates (±standard errors).

differences in translation between the two plant species became clearer with increasing extent of substitution (Fig. 3). No significant decrease in ^{35}S-methionine incorporation was found for sugar beet up to a K^+ substitution level of 80%.

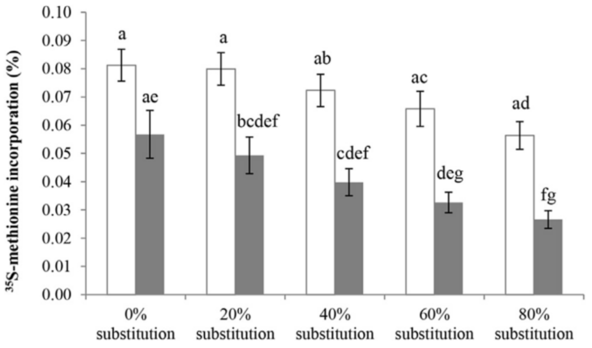

Fig. 3. Effect of different substitution of K^+ by Na^+ on the relative *in vitro* incorporation of ^{35}S-methionine into translation products by polysomes isolated from sugar beet (white columns) and maize (grey columns). Reactions were performed in the presence of 6 mM Mg^{2+}. 126 mM K^+ (0% substitution) was stepwise substituted by Na^+ (20% substitution = 100.8 mM K^+, 25.2 mM Na^+, 40% substitution = 75.6 mM K^+, 50.4 mM Na^+, 60% substitution = 50.4 mM K^+, 75.6 mM Na^+, and 80% substitution = 25.2 mM K^+, 100.8 mM Na^+). *In vitro* translation assays were prepared as described in Material and methods. Results are expressed as means ± standard errors. Means were calculated from four biological replicates. The mean of each biological replicate was obtained from three technical replicates. Two-way ANOVA with post-hoc analysis (Tukey-HSD) was carried out by means of Statistica (Version 12, Tulsa, Oklahoma, USA). Significant differences ($P < 0.05$) are indicated by different letters.

In order to elucidate the effect of the substitution on *in vitro* translation, the incorporation of ^{35}S-methionine into translation products was expressed relatively to the control (0% substitution) (Fig. 4). This relative expression compensates for differences in activity between polysomes of sugar beet and maize and within the biological replicates. Whereas the inhibitory effect of the substitution on *in vitro* translation of sugar beet polysomes was not statistically significant when the absolute translation data (Fig. 3) were considered, the relative expression showed that translation of both plant species was inhibited by the substitution of Na^+ by K^+ (Fig. 4). However, the *in vitro* translation of maize polysomes was more negatively affected by the substitution compared to sugar beet. A significant decrease in the translation of maize polysomes was observed when 20% of K^+ were replaced by Na^+, whereas in the case of sugar beet, the translation was inhibited at the substitution level of 40% (Fig. 4). Also, at higher substitution levels translation was relatively more inhibited in maize polysomes than in sugar beet polysomes (Fig. 4).

4. Discussion

It was shown that net protein synthesis is the process which is most sensitive to the substitution of K^+ by Na^+ in leaves of young sugar beet (Faust and Schubert, 2016). However, translation was not inhibited due to a shortage of amino acids. The concentrations of

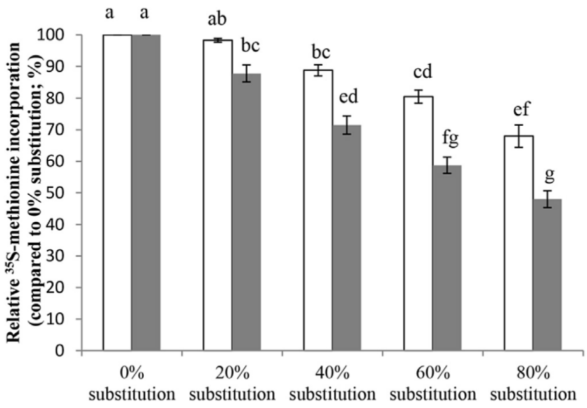

Fig. 4. Effect of different substitution of K^+ by Na^+ on the relative *in vitro* incorporation of ^{35}S-methionine into translation products by polysomes isolated from sugar beet (white columns) and maize (grey columns). Reactions were performed in the presence of 6 mM Mg^{2+}. 126 mM K^+ (0% substitution) was stepwise substituted by Na^+ (20% substitution = 100.8 mM K^+, 25.2 mM Na^+, 40% substitution = 75.6 mM K^+, 50.4 mM Na^+, 60% substitution = 50.4 mM K^+, 75.6 mM Na^+, and 80% substitution = 25.2 mM K^+, 100.8 mM Na^+). *In vitro* translation assays were prepared as described in Material and methods. Data obtained for the substitution treatments are expressed as means ± standard errors relative to the control (0% substitution). Means were calculated from four biological replicates. The mean of each biological replicate was obtained from three technical replicates. Two-way ANOVA with post-hoc analysis (Tukey-HSD) was carried out by means of Statistica (Version 12, Tulsa, Oklahoma, USA). Significant differences ($P < 0.05$) are indicated by different letters.

each single free proteinogenic amino acid even increased with the extent of the substitution. Therefore, we hypothesized that the activity of ribosomes is inhibited due to the substitution of K^+ by Na^+. In order to test this hypothesis, polysomes from leaves sugar beet and maize were isolated and the effect of the substitution of K^+ by Na^+ on protein synthesis was tested *in vitro*.

4.1. Suitability of the in vitro translation systems from sugar beet and maize

Before the isolated polysomes were used in the substitution experiments, the functionality and comparability of both *in vitro* systems was tested by means of the sequential omission of reaction mixture components (Table 1). Both systems showed an absolute requirement of polysomes for ^{35}S-methionine incorporation. ^{35}S-methionine incorporation was dependent on the amount of added polysomes (Table 1). The fact that only 2% and 3% of the activity were found in the absence of polysomes in the *in vitro* systems of sugar beet and maize, respectively, showed that the deletion of ribosomes from wheat germ lysate by means of centrifugation according to Stupina and Simon (2013) was successful. Hence, the contribution of wheat germ ribosomes to the formation of translation products could be excluded.

Both systems responded similarly to the omission of the supernatant: The incorporation of ^{35}S-methionine decreased to about one-fifth of the incorporation of complete system (Table 1). This allows the conclusion that under the chosen conditions of polysome isolation translation factors were not completely washed from plant ribosomes and that the stimulation of ^{35}S-methionine incorporation by translation factors from the wheat germ supernatant was comparable for the two systems. Like other *in vitro* translation systems (Boulter, 1970) both tested systems relied on the supply of metabolic energy and the presence of an energy-regenerating system (Table 1).

Amino acid incorporation into translation products was slightly increased due to the omission of chloramphenicol (+8% for sugar beet and +18% for maize) (Table 1). This increase resulted from ^{35}S-methionine incorporation by ribosomes from chloroplasts, mitochondria and/or from bacterial contamination, whose activity is inhibited by chloramphenicol (Anderson and Smillie, 1966; Huang et al., 1966), and was in a similar range as found for *in vitro* systems of polysomes from leaves of glycophytes and halophytes (Flowers and Dalmond, 1992). Although the contamination with non-cytosolic ribosomes was not high, chloramphenicol was included in further experiments to exclude a contamination effect.

The lack of requirement of exogenous supply of amino acids (Table 1) indicates that amino acids were associated with isolated ribosomes or may arise from creatine phosphokinase preparation (Boulter, 1970). Translation of both *in vitro* systems was not inhibited due to the omission of exogenous mRNA (Table 1). This indicates that *in vitro* translation was not limited by endogenous mRNA. Nevertheless, exogenous amino acids and mRNA were supplied to *in vitro* translation systems to guarantee that *in vitro* translation was not limited by substrate shortage and was only dependent on the activity of cytosolic polysomes.

4.2. Different Mg^{2+} and K^+ requirements of the two species

Before the effect of the substitution of K^+ by Na^+ was tested, it was necessary to identify Mg^{2+} and K^+ concentrations which enable proper functioning of *in vitro* translation of both plant species. Both cations contribute to the structural stability of ribosomal RNA and ribosomal proteins and are needed for the integrity, assembly, and the functionality of ribosomes (Klein et al., 2004).

For the polysomes from maize leaves *in vitro* translation was highest at Mg^{2+} concentrations of 4–6 mM and K^+ concentrations of 101–126 mM (Figs. 1 and 2). These concentrations are close to the *in vitro* requirements of polysomes isolated from leaves of several glycophytes (*Pisum sativum*, *Oryza sativa*, *Hordeum vulgare*, *Secale cereale*). The optimal Mg^{2+} and K^+ supply for these *in vitro* systems was 2.5–4 mM and 125–150 mM, respectively (Flowers and Dalmond, 1992; Laroche and Hopkins, 1987). *In vitro* translation of sugar beet polysomes was highest at Mg^{2+} concentrations of 6–8 mM and a K^+ concentration of 151 mM (Figs. 1 and 2). These results are consistent with the results of Flowers and Dalmond (1992). They showed higher Mg^{2+} and K^+ requirements for polysomes from halophytes compared to glycophytes. *In vitro* systems of *Atriplex isatidea*, *Inula crithmoides*, and *Suaeda maritima* required 6–8 mM Mg^{2+} and 125–275 mM K^+ for maximum amino acid incorporation.

In order to be able to compare the two *in vitro* systems with regard to their sensitivity to the substitution of K^+ by Na^+, the same ion concentrations, which meet the requirements of both plant species, must be present in the reaction assay. Therefore, Mg^{2+} and K^+ were offered at concentrations of 6 mM and 126 mM in the substitution experiments. Both systems showed high translational activity at these concentration levels (Figs. 1 and 2). The K^+ concentration of 126 mM and the Mg^{2+} concentration of 6 mM chosen for further *in vitro* experiments reflect the estimated cytosolic *in vivo* K^+ concentrations of 100–200 mM and Mg^{2+} concentrations of 2–10 mM for plants grown under non-saline conditions (Leigh and Wyn Jones, 1986).

4.3. Different substitution tolerance of the two species

^{35}S-methionine incorporation of both *in vitro* systems was inhibited by the substitution of K^+ by Na^+ (Fig. 4). This is in line with the observed inhibition of translation of prokaryotic and eukaryotic *in vitro* systems (e.g. Gibson et al., 1984; Lubin and Ennis, 1964) under substitution conditions. This indicates that the process of translation itself is disturbed due to the substitution and that Na^+

cannot fulfill completely specific functions of K^+ in translation. K^+ is essential for the native conformation of ribosomes (Klein et al., 2004; Näslund and Hultin, 1970), translation factors, and enzymes involved in the translation process. Therefore, initiation (Spyrides, 1964), elongation (Lubin and Ennis, 1964) as well as termination (Näslund and Hultin, 1971) of translation are dependent on K^+. However, the precise site(s) of inhibition due to Na^+ toxicity and/or K^+ deficiency is/are not known yet and was/were not further investigated in the present study.

However, the extent of inhibition was different for the two tested *in vitro* systems: Translation of polysomes of sugar beet was less negatively affected by the substitution compared to maize polysomes (Figs. 3 and 4). One possible reason for the higher substitution tolerance of the sugar beet polysomes could be that the ribosomal proteins of sugar beet are less susceptible to conformational changes than the ribosomal proteins of maize. Analysis of cytosolic ribosomes of *Arabidopsis thaliana* revealed high heterogeneity within the ribosomal proteins (Giavalisco et al., 2005). Many ribosomal proteins are present in different isoforms and/or are modified due to posttranslational modifications (Carroll, 2013; Giavalisco et al., 2005). Therefore, it is very likely that differences exist in the ribosomal protein composition in sugar beet and maize, which could explain the different sensitivity towards the substitution of K^+ by Na^+.

It is possible that differences in susceptibility to conformational changes exist not only for ribosomal proteins, but also for translation factors of sugar beet and maize, which could explain the improved tolerance of the sugar beet *in vitro* system. The results of Rausell et al. (2003) support this possibility. Overexpression of the sugar beet translation initiation factor *BveIF1A* increased salt resistance of yeast and Arabidopsis. *eIF1A* together with *eIF1* induces an open conformation of the 40 S ribosome (Passmore et al., 2007). This conformational status of the small ribosomal subunit allows mRNA and ternary complex binding and is therefore necessary for the further progress of translation initiation. Moreover, the authors showed that *BveIF1A* stimulated *in vivo* protein synthesis in yeast and *in vitro* protein synthesis of wheat germ system under saline conditions. Rausell et al. (2003) showed that *BveIF1A* is expressed in leaves of three-week old sugar beet plants in the absence of salt stress. Therefore, we can assume that this initiation factor may also be found in the sugar beet plants of this study. When the wheat germ supernatant was omitted from the *in vitro* reaction assay (Table 1), the remaining ^{35}S-methionine incorporation of 22% and 16% of the sugar beet and maize system, respectively, indicates that translation factors were associated with the isolated polysomes. Sequence alignment of *BveIF1A* with its maize homologs revealed that five amino acids are altered within the oligonucleotide binding site (not shown), which could explain differences in susceptibility to conformational changes. It is possible that the conformation of *BveIF1A* is not affected by high Na^+/K^+ ratios, in contrast to its homologs in maize, and is able to fulfill its role in translation initiation under substitution conditions.

In our *in vitro* substitution experiments, K^+ and Na^+ were provided in a range from 126 mM down to 25 mM and from 0 mM up to 101 mM, respectively. Unfortunately no data are reported which allow the assessment of the cytosolic concentrations of K^+ and Na^+ in leaves of sugar beet and maize under saline conditions. Munns and Tester (2008) estimated that Na^+ concentrations are kept low at a level of 10–30 mM, whereas cytosolic K^+ concentrations in range of 80–100 mM are likely under saline conditions. These estimated *in vivo* concentrations are reflected by the *in vitro* substitution treatment of 20% (101 mM K^+; 25 mM Na^+). As mentioned above, this substitution level inhibited *in vitro* translation of maize polysomes, but no inhibition was observed for translation of sugar beet polysomes (Fig. 4). Surprisingly, increasing the level of substitution up to 80% (25 mM K^+; 101 mM Na^+), which means a more than threefold higher *in vitro* Na^+ concentration than expected *in vivo*, did not result in a total inhibition of translation neither for sugar beet nor for maize polysomes: Compared to control conditions 68% and 48% of ^{35}S-methionine incorporation were measured for sugar beet and maize polysomes, respectively (Fig. 4). This lack of inhibition may be related to the requirement of a high ionic concentration for protein stability that is provided by the high charge density of membrane complexes or multi protein complexes found in the cytosol *in vivo* (Cheeseman, 2013).

4.4. Constitutive adaptation of key processes in sugar beet to high Na^+/K^+

Since several cytosolic enzymes from halophytes were similarly sensitive to high Na^+ concentrations as those from glycophytes (Flowers, 1972; Greenway and Osmond, 1972), the conclusion was drawn that the cellular basis of salt resistance in halophytes depends upon the successful compartmentalization of ions in vacuoles and the osmotic adjustment of the cytosol by means of compatible solutes (Flowers, 1985).

Due to the findings of Flowers (1972) and Greenway and Osmond (1972) a general adaptation of halophytic metabolism to high cytosolic Na^+/K^+ was excluded. However, the increased *in vitro* tolerance of sugar beet polysomes towards the substitution of K^+ by Na^+ compared to polysomes from maize (Figs. 3 and 4), indicates an *in vivo* adaptation of translation in halophytes to increased Na^+ concentrations and Na^+/K^+ ratios. This finding indicates that the salt resistance in sugar beet is not only due to a successful compartmentalization of Na^+. The polysomes were obtained from sugar beet plants which had been grown under control conditions, hence, the adaptation of sugar beet polysomes to high Na^+/K^+ was constitutive.

The concept of a constitutive adaptation of key processes in sugar beet is supported by the results of Wakeel et al. (2011). They showed that the plasma membrane (PM) H^+-ATPase of sugar beet is less affected by the substitution of K^+ by Na^+ than the PM H^+-ATPase of maize. This key enzyme enables essential processes in plants such as nutrient uptake, phloem loading, pH regulation, and extension growth (Palmgren, 2001). It was shown *in vitro* that the hydrolytic activity of the sugar beet PM H^+-ATPase was even not impaired by a complete substitution of K^+ by Na^+ (Wakeel et al., 2011), whereas the hydrolytic activity of maize PM H^+-ATPase was already significantly reduced at a substitution level of 25%. The proton pumping activity of the PM H^+-ATPase from sugar beet was also less affected by the substitution than that of maize (Wakeel et al., 2011). Similar tolerance of PM H^+-ATPase of sugar beet plants, which were grown with and without salt stress, indicates that the tolerance of this enzyme is also constitutive. The difference in translation product formation between sugar beet and maize polysomes became more prominent with increasing extent of the substitution (Figs. 3 and 4), this is also in line with the results of Wakeel et al. (2011). They showed that the activity of PM H^+-ATPase of maize was relatively more inhibited than that of sugar beet at higher substitution levels.

5. Conclusions

We conclude that the results of the present study and of Wakeel et al. (2011) indicate a constitutive adaptation of special key processes to increased Na^+ concentrations or Na^+/K^+ ratios. We propose that the salt resistance of sugar beet is not only realized by efficient ion compartmentalization but also by adaptation of key enzymes.

Author contribution

SS designed the study. FF conducted experiments and analyzed data. SS and FF wrote the manuscript.

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4 Discussion

It is well known that K^+ can be replaced by Na^+ to a great extent in *Beta vulgaris*. However, it was not clear which process limits the substitution of K^+ by Na^+ in young sugar beet. Therefore, sugar beets were grown at various substitution levels (Faust and Schubert, 2016). The sum of supplied K^+ and Na^+ concentrations was kept constantly equal at the level of adequate K^+ nutrition of 4 mM. The approach excluded chloride or osmotic effects.

4.1 Net protein synthesis is the most sensitive process under substitution of K^+ by Na^+ in young sugar beet

Opening and closing of stomata enable gas exchange and transpiration. These stomatal movements are facilitated by reversible K^+ fluxes (Robinson et al., 1997). It was shown that Na^+ can replace K^+ in the opening of stomata in epidermal strips of *Vicia faba* and *Commelina communis* (Humble and Hsiao, 1969; Jarvis and Mansfield, 1980). However, the substitution of K^+ by Na^+ inhibited closing of stomata (Jarvis and Mansfield, 1980). K^+ channels in the plasma membrane (PM) of guard cells of *Vicia faba* are blocked by the presence of Na^+ (Thiel and Blatt, 1991). Since guard cells represent “watergates” (Roelfsema and Hedrich, 2005), it was suggested that Na^+-induced inhibition of stomata closing was responsible for uncontrolled water losses leading to the appearance of spot necrosis in *Vicia faba* (Slabu et al., 2009). Therefore, it was hypothesized that transpiration of young sugar beet plants is the process which is most sensitive to the substitution of K^+ by Na^+ (Faust and Schubert, 2016). However, the process of transpiration was not affected by the substitution. The transpiration rate was not increased even when 99.75% of K^+ were substituted by Na^+ (Faust and Schubert, 2016; Supplemental Figure 1). Therefore, this hypothesis was rejected.

All three key functions of K^+ are required for processes involved in plant growth. K^+ is needed for the activation of enzymes such as the PM H^+-ATPase (Gibrat et al., 1990) which plays an important role in extension growth (Hager et al., 1971). Potassium serves for charge balance during sucrose loading into the phloem which feeds young growing tissue (Cakmak et al., 1994). Moreover, as an osmotic agent K^+ contributes as an osmotic agent to the generation of turgor (Mengel and Arneke, 1982). This list of examples for the involvement of K^+ in growth processes is incomplete. Due to the multiple functions of K^+ in plant growth, it was hypothesized that growth is the process which is most sensitive to the substitution of K^+ by Na^+ in young sugar beet (Faust and Schubert, 2016). The growth of young sugar beet plants was inhibited under the substitution of 99.75% K^+ by Na^+ (Faust and Schubert, 2016; Figure 5 B, D). However, sugar concentrations and protein contents were significantly reduced when 98.75% and 97.50% of K^+ were substituted by Na^+ in the nutrient solution, respectively (Faust and Schubert, 2016; Figure 6 D, Figure 7 B). Therefore, growth was not the most sensitive process during the substitution of K^+ by Na^+ in sugar beet. The hypothesis was rejected.

A sufficient supply of K^+ is needed for the process of translation (Wyn Jones et al., 1979). Potassium is needed for the stability and functionality of ribosomes (Conway, 1964; Lubin and Ennis, 1964; Näslund and Hultin, 1970). Hence, the hypothesis was set up that net protein synthesis is the most sensitive process during the substitution of K^+ by Na^+ (Faust and Schubert, 2016). Protein content per shoot was chosen as a parameter for the quantification of the effect of the substitution on net protein synthesis. Protein contents were reduced when 97.50% of K^+ were substituted by

Na^+ (Faust and Schubert, 2016; Figure 7 B). Transpiration, growth, and the supply of sugars were not inhibited by this substitution level (Faust and Schubert, 2016; Figure 5 B, D; Figure 6 D; Supplemental Figure 1 B). Therefore, this hypothesis was accepted.

4.2 The process of translation itself is inhibited due to the substitution of K^+ by Na^+

Besides K^+, Mg^{2+} plays an important role for the structure and activity of ribosomes (Gesteland, 1966;Cammarano et al., 1972). The inhibition of protein synthesis by Mg^{2+} deficiency in young sugar beet was excluded for the conducted substitution experiments (Faust and Schubert, 2016; Figure 4 A,F). Moreover, the inhibition of translation by a shortage of proteinogenic amino acids was excluded (Faust and Schubert, 2016; Table 2). Free amino acids even accumulated with increasing the extent of the substitution. Therefore, it was hypothesized that the process of translation itself is inhibited due to the substitution of K^+ by Na^+ (Faust and Schubert, 2017).

In order to test this hypothesis, ribosomes were isolated from growing leaves of sugar beet and maize which were grown under control conditions (Faust and Schubert, 2017). In an *in vitro* approach the translation of the ribosomes from both plant species was quantified via the incorporation of ^{35}S-methionine in peptides and proteins. In order to avoid the translation of ribosomes of plastids and mitochondria, organellar ribosomes were inhibited by means of the addition of chloramphenicol to both *in vitro* systems. The optimal Mg^{2+} and K^+ concentrations were determined for ribosomes of both plant species. Mg^{2+} and K^+ concentrations were defined which meet the requirements of both *in vitro* systems. K^+ was substituted by Na^+ at different levels (0%, 20%, 40%, 60%, and 80% substitution). The effect of different substitution levels on the ^{35}S-methionine incorporation of both *in vitro* systems was determined (Faust and Schubert, 2017).

The substitution of K^+ by Na^+ inhibited the *in vitro* translation of both plant species (Faust and Schubert, 2017; Figure 4). This shows that the process of translation itself is inhibited by the substitution and explains the observed inhibition of net protein synthesis *in vivo* under substitution conditions (Faust and Schubert, 2016). Therefore, the hypothesis was accepted.

4.3 Sugar beet ribosomes are less sensitive to the substitution of K^+ by Na^+ than ribosomes from maize

Results of Wakeel et al. (2011) showed in *in vitro* experiments that the PM H^+-ATPase of sugar beet is more tolerant to high Na^+/K^+ ratios than the enzyme from maize. The PM H^+-ATPase is a key enzyme in plants (Palmgren, 2001). Also ribosomes play a key role in the metabolism of plants. Based on the results of (Wakeel et al., 2011), it was hypothesized that the ribosomes of sugar beet are less susceptible to the substitution of K^+ by Na^+ than the ribosomes of maize (Faust and Schubert, 2017).

Ribosomes of maize responded more sensitively to the substitution of K^+ by Na^+ than the ribosomes of sugar beet (Faust and Schubert, 2017; Figure 3 and 4). *In vitro* translation of maize ribosomes was inhibited by the substitution level of 20%, whereas *in vitro* translation of the sugar beet ribosomes was reduced when 40% of K^+ were substituted by Na^+ (Faust and Schubert, 2017; Figure 4). Hence, the hypothesis was accepted.

4.4 Is the dogma of Greenway and Munns (1980) still valid?

In 1980 a dogma of salt resistance of plants was raised by Grennway and Munns. According to this dogma (Greenway and Munns, 1980) the high level of the salt resistance of halophytes is based on the successful inclusion of salts. This compartmentalization of ions in vacuoles and the synthesis of compatible solutes enable halophytes to achieve a higher level of salt resistance than glycophytes. The lower level of the salt resistance of glycophytes was ascribed to the inadequate sequestration of ions in leaves. The tolerance of halophytic enzymes to elevated Na^+ (and Cl^-) concentrations was not considered to be higher than those of glycophytic plants. Therefore, the contribution of tolerant enzymes to the salt resistance of halophytes was regarded as unlikely (Greenway and Munns, 1980).

It is difficult to make a clear distinction between glycophytes and halophytes. The definition of glycophytes and halophytes is a vigorously debated issue (Flowers and Colmer, 2008). Plants show a continuum in their level of salt resistance (Flowers and Colmer, 2015). Flowers et al. (1977) defined halophytes as plants which are able to complete their life cycle in the presence of 300 mM (or higher) salt (predominantly NaCl). The characterization of maize as a glycophyte and sugar beet as a halophyte corresponds to this definition: Sugar beet keeps up growth under a salt stress of 500 mM NaCl (Papp et al., 1983), whereas some maize genotypes cannot survive a salt stress of 200 mM NaCl (Hajibagheri et al., 1987). However, this definition of glycophytes and halophytes is very rough. Halophytes are found in at least 37 orders of plants (Cheeseman, 2013). Many halophytes, such as sugar beet, belong to the family of *Amaranthaceae*. The growth response of halophytic plants to different salts is quite diverse. The optimal growth of halophytes was observed in a range from 20 to 500 mM NaCl (Flowers et al., 1977). Moreover, the habitats of halophytes may not be only high in NaCl, but may also contain other soluble salts such as KCl at high concentrations. The response of halophytes to KCl is case-specific. Some halophytes show a similar growth response under KCl treatment as with NaCl. In contrast, KCl seems to be toxic for other halophytes (Flowers et al., 1977).

The exclusion of salts was characterized as the response of glycophytic plants to salt stress (Flowers et al., 1977). However, the strategies of passive and/or active exclusion are also used by halophytes (Munns, 2002). Patch-clamp experiments showed that the selectivity for K^+ over Na^+ of inward-rectifying K^+ channels is much higher in the root cells of the halophyte *Thellungiella halophila* compared to its glycophytic relative *Arabidopsis thaliana* (Volkov et al., 2004). Also active exclusion is up-regulated in some halophytes due to salt stress: Na^+/H^+ antiporter *SOS1* activity was increased in the PM vesicles of the halophyte *Atriplex nummularia* when plants were grown in the presence of NaCl (Hassidim et al., 1990). Also Oh et al. (2009) demonstrated the importance of *SOS1* for the salt resistance of *Thellungiella halophila*. The salt-induced expression of *SOS1* in the leaves of the halophyte *Mesembryanthemum crystallinum* indicates that this Na^+/H^+-antiporter (*McSOS1*) plays a role in the avoidance of Na^+ accumulation in photosynthetic leaf cells (Cosentino et al., 2010). The authors suggested that *McSOS1* directs Na^+ to specialized epidermal bladder cells. Moreover, salt resistance of glycophytes is not always determined by the ability to exclude Na^+ (Munns and Tester, 2008). There is no correlation between Na^+ concentrations in leaves and the salt resistance of bread and durum wheat genotypes (Munns and James, 2003; Genc et al., 2007).

According to the dogma of Greenway and Munns (1980) the compartmentalization of ions in vacuoles is characterized as a strategy of halophytes against salt stress. However, this strategy is also found in glycophytes: The salt stress-induced expression of Na^+/H^+ antiporters *NHX* (proven at the transciptional level) contributes to the salt resistance of some maize and wheat genotypes (Saqib et al., 2005; Pitann et al., 2013).

The dogma of Greenway and Munns (1980) also excluded an adaptation of halophytic enzymes to elevated Na^+ (and Cl^-) concentrations. This conclusion of Greenway and Munns (1980) was based on the results of Jennings (1976) and studies reviewed in Flowers et al. (1977). These authors concluded from results of their *in vitro* studies that the enzymes of glycophytes and halophytes are similarly sensitive to NaCl. These (and other) studies, which compared the effect of NaCl on enzymes from halophytes and glycophytes, followed *in vitro* Approach 1 (Figure 2). This approach is based on an insufficient K^+ supply of the enzyme. It is possible to test whether Na^+ can compensate the deficiency of K^+ by adding of NaCl to the reaction medium. However, it is important to consider that the electrolyte concentration and the Na^+/K^+ ratio change due to the addition of NaCl. Moreover, enzyme activity is influenced by additional chloride and osmotic effects.

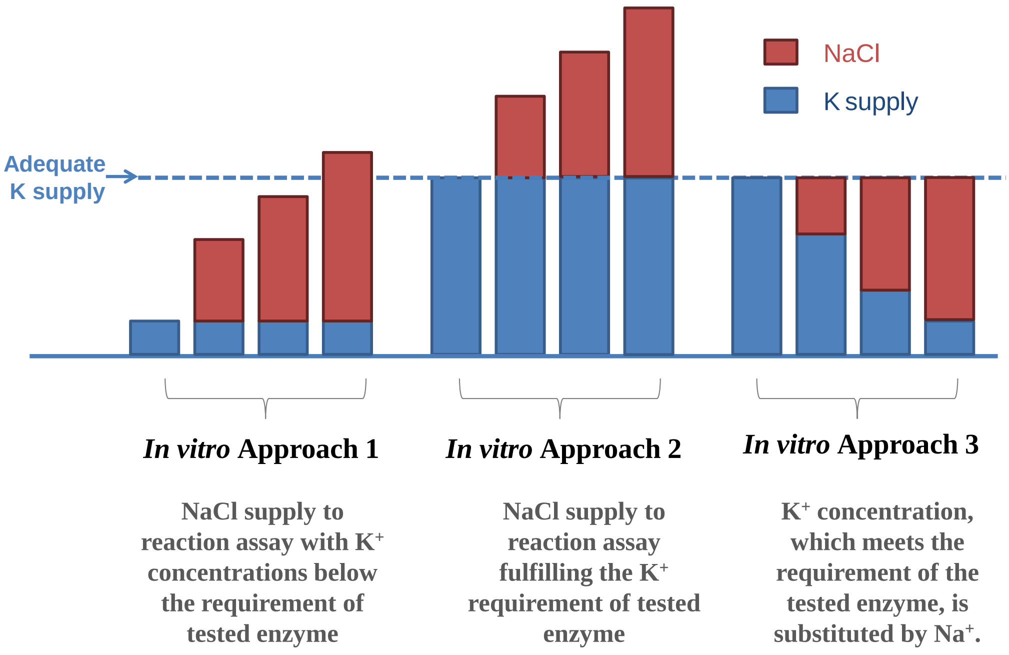

Figure 2: Schematic illustration of *in vitro* approaches used to compare corresponding enzymes from halophytes and glycophytes.

Many *in vitro* studies following this first approach observed a stimulation of enzyme activity in halophytes and glycophytes under low NaCl conditions (Flowers, 1972; Greenway and Osmond, 1972; von Willert, 1974; Flowers et al., 1976; Flowers and Dalmond, 1992). This stimulation indicates that Na^+ can at least partially replace K^+ in the activation of halophytic and glycophytic enzymes. However, the extent of the stimulation of enzyme activity by low Na^+ concentrations differed significantly for some corresponding glycophytic and halophytic enzymes (malate dehydrogenase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, *in vitro* protein synthesis) (Greenway and Osmond, 1972; Flowers and Dalmond, 1992). The stimulation of soluble malate dehydrogenase from the leaves of *Atriplex spongiosa* by 50 mM NaCl was significantly higher (+80% stimulation) than that of *Phaseolus vulgaris* (+20% stimulation) (Greenway and Osmond, 1972). Flowers and Dalmond (1992) showed that the *in vitro* translation of ribosomes from *Pisum sativum* increased by 18% when 100 mM Na-acetate were added to the reaction medium (containing 25 mM K-acetate). The same treatment increased the *in vitro* translation of ribosomes from the halophyte *Inula crithmoides* by 620% (Flowers and Dalmond, 1992). These results indicate that some halophytic enzymes are better able to use Na^+ for enzyme activation (in the absence of sufficient K^+) than those of glycophytes.

Halophytic and glycophytic corresponding enzymes showed a similar inhibition of enzyme activity due to high NaCl concentrations (Flowers, 1972; Greenway and Osmond, 1972; Austenfeld, 1976). Due to multiple effects (the increase of ionic strength, the change of Na^+/K^+ ratio, the increase of osmotic effects, the increase of chloride effects), the possible differences in the activation of halophytic and glycophytic enzymes by Na^+ are masked.

In order to evaluate the effect of salt stress on enzyme activities, the activities in absence of NaCl are used as references in many *in vitro* studies (e.g. Flowers, 1972; Osmond and Greenway, 1972; von Willert, 1974). However, the comparison of the effect of NaCl on corresponding glycophytic and halophytic enzymes is only possible when the enzymes exhibit similar requirements for the supply of K^+. Flowers and Dalmond (1992) showed that halophytic ribosomes require higher K^+ concentrations than ribosomes of glycophytes. In extreme cases, such as for ribosomes from *Oryza sativa* and *Inula crithmoides*, the difference in optimal K^+ concentrations could be as high as 175 mM (Flowers and Dalmond, 1992). This means that the absence or the insufficient supply of K^+ to the reaction medium represents very different reference conditions for the enzymes and hence, the comparability of the enzymes with this experimental approach is questionable.

Therefore, it is important to guarantee an optimal supply of K^+ (see Figure 2; *in vitro* approach 2). It is possible to test whether enzymes are able to cope with high Na^+

(and Cl^-) concentrations, by using this *in vitro* approach. However, as *in vitro* Approach 1, changes in Na^+/K^+ ratios and osmotic effects occur upon NaCl application and could affect enzyme activity. It was shown that an excess of acidic amino acids over basic amino acids provides stability and functionality to malate dehydrogenase of the halophilic archaeon *Haloarcula morismortui* (Dym et al., 1995). Dym et al. (1995) suggested that the acidic residues are able to bind more water molecules than other amino acid residues under physiological pH conditions. The formation of a protective hydration sphere could be responsible for the lack of protein aggregation under highly saline conditions (Dym et al., 1995). Also ribosomal proteins of *Haloarcula morismortui* show higher concentrations of aspartate and glutamate than ribosomal proteins of non-halophilic eukaryotes and eubacteria (Kimura et al., 1989).

In the third *in vitro* approach (Figure 2) K^+ is substituted by Na^+. Potassium is supplied in sufficiently high concentrations to meet the requirements of the tested enzymes. The sum of supplied K^+ and Na^+ is kept constant. Due to the substitution of K^+ by Na^+, the Na^+/K^+ ratio is changed. In contrast to the *in vitro* Approaches 1 and 2 (Figure 2), osmotic and chloride effects are excluded in this third approach. This experimental approach allows testing whether glycophytic and halophytic enzymes differ in their ability to use Na^+ (instead of K^+) for enzyme activation. Plasma membrane H^+-ATPase as well as ribosomes of sugar beet were less sensitive to the substitution of K^+ by Na^+ than those of maize (Wakeel et al., 2010, 2011; Faust and Schubert, 2017). The superior Na^+ tolerance of these key enzymes of sugar beet could be accomplished by the expression of specific isoforms or post-translational modifications of PM H^+-ATPase and ribosomal proteins/translation factors. Contrary to the dogma of Greenway and Munns (1980), the contribution of the tolerance of key enzymes to the salt resistance of halophytes is thus likely.

Uncertainties exist in the transfer of results of *in vitro* studies to *in vivo* conditions. The *in vitro* Approaches 2 and 3 investigate different aspects of salt stress in a simplified form. It has to be considered that the sensitivity of enzymes is influenced by changes in the substrate concentration, the enzyme concentration, or the choice of the extraction method (Flowers et al., 2015). Shomer-Ilan et al. (1991) showed that the increase of phosphoenolpyruvate concentration reduced the *in vitro* sensitivity of maize pyruvate kinase to NaCl. It was shown *in vitro* that compatible solutes such as glycinebetaines and other proline analogues were able to reduce the sensitivity of pyruvate kinase and malate dehydrogenase from maize and barley to NaCl (Pollard and Wyn Jones, 1979; Shomer-Ilan et al., 1991). Moreover, enzymes which are present as protein complexes *in vivo* can exhibit a different level of sensitivity than in a highly diluted and separated form *in vitro* (Cheeseman, 2013).

5 Conclusions

It is concluded that the dogma of salt resistance by Greenway and Munns (1980) is not valid. The salt resistance of halophytes is not only based on the successful compartmentalization of ions, but also on the increased level of Na^+ tolerance of key enzymes compared to those of glycophytes. It was shown in the literature that glycophytes also use the strategy of sequestration of ions in vacuoles. Halophytes and glycophytes do not differ in regard to their qualitative strategies, but in quantitative aspects.

6 Summary

It is well known that potassium (K^+) can be replaced by sodium (Na^+) to a great extent in sugar beet (*Beta vulgaris)*. However, the possible extent of substitution is limited also in sugar beet. It was not clear which process limits the substitution of K^+ by Na^+ in young sugar beet. It is known that K^+ is required for the processes of transpiration, growth, and protein synthesis. The aim was to find out which process is most sensitive due to the substitution of K^+ by Na^+.

Therefore, sugar beets were grown at various substitution levels (0.25%, 25.00%, 50.00%, 75.00%, 97.50%, 98.75%, and 99.75% substitution). The sum of supplied K^+ and Na^+ concentrations was kept constantly equal at the level of adequate K^+ nutrition of 4 mM. The approach excluded chloride or osmotic effects.

The process of transpiration was not affected by the substitution. The transpiration rate was not increased even when 99.75% of K^+ were substituted by Na^+. The growth of young sugar beet plants was inhibited under the substitution of 99.75% K^+ by Na^+. The supply of sugars was inhibited when 98.75% of K^+ were substituted by Na^+. Protein content per shoot was chosen as a parameter for the quantification of the effect of the substitution on net protein synthesis. Protein contents were reduced when 97.50% of K^+ were substituted by Na^+. Since transpiration, growth, and the supply of sugars were not inhibited by this substitution level, net protein synthesis was the most sensitive process during substitution of K^+ by Na^+ in young sugar beet.

The inhibition of net protein synthesis due to a substitution-induced magnesium (Mg^{2+}) deficiency or due to a shortage of proteinogenic amino acids was excluded. Free amino acids even accumulated with increasing the extent of the substitution.

Therefore, it was hypothesized that the process of translation itself is inhibited due to the substitution of K^+ by Na^+.

In order to test this hypothesis, ribosomes were isolated from growing leaves of sugar beet and maize which were grown under control conditions. In an *in vitro* approach the translation of the ribosomes from both plant species was quantified via the incorporation of ^{35}S-methionine in peptides and proteins. In order to avoid the translation by ribosomes of plastids and mitochondria, organellar ribosomes were inhibited by means of the addition of chloramphenicol to both *in vitro* systems. Magnesium and K^+ concentrations were identified which meet the requirements of both *in vitro* systems. Potassium was substituted by Na^+ at different levels (0%, 20%, 40%, 60%, and 80% substitution). The effect of different substitution levels on the ^{35}S-methionine incorporation of both *in vitro* systems was determined.

The substitution of K^+ by Na^+ inhibited the *in vitro* translation of both plant species. This shows that the process of translation itself is inhibited by the substitution and explains the observed inhibition of net protein synthesis *in vivo* under substitution conditions. However, the ribosomes of sugar beet exhibited higher level of tolerance than the ribosomes of maize.

Besides the plasma membrane H^+-ATPase, the ribosomes represent the second example for increased tolerance of high Na^+/K^+ ratios in the metabolism of sugar beet. This indicates that the salt resistance of sugar beet is not only based on the successful compartmentation of ions in vacuoles, but also on the Na^+ tolerance of key enzymes.

7 Zusammenfassung

Es ist schon lange bekannt, dass die Zuckerrübe (*Beta vulgaris*) gut mit Natrium (Na^+) zurechtkommt. Allerdings ist auch bei der Zuckerrübe keine vollständige Substitution von Kalium (K^+) durch Na^+ möglich. Bisher war nicht klar, welcher Prozess am empfindlichsten gegenüber der Substitution reagiert und somit die Substitution limitiert. Kalium spielt eine bedeutende Rolle bei der Regulation der Transpiration, bei Wachstumsprozessen und bei der Proteinsynthese. Ziel dieser Arbeit war, den Prozess zu identifizieren, der am empfindlichsten gegenüber der Substitution von K^+ durch Na^+ reagiert.

Dazu wurden Zuckerrüben in verschiedenen Substitutionsvarianten (0,25%, 25,00%, 50,00%, 75,00%, 97,50%, 98,75% und 99,75% Substitution) in Wasserkultur angezogen. Die Summe der K^+- und Na^+-Konzentrationen wurde dabei in jeder Variante konstant bei 4 mM gehalten. Dieser Versuchsansatz wurde gewählt, um zusätzliche Einflüsse einer Akkumulation von Chloridionen oder osmotische Einflüsse auszuschließen.

Der Prozess der Transpiration wurde nicht von der Substitution beeinträchtigt. Selbst bei einer nahezu vollständigen Substitution von K^+ durch Na^+ (99,75% Substitution) konnte kein Anstieg der Transpirationsrate festgestellt werden. Das Wachstum der jungen Zuckerrübenpflanzen wurde erst beeinträchtigt, wenn 99,75% des K^+ durch Na^+ ersetzt wurden. Die Bereitstellung von Zuckern, eine wichtige Voraussetzung für die Versorgung von jungem, wachsendem Gewebe, wurde durch die Substitution von 98,75% des K^+ durch Na^+ gestört. Proteingehalte wurden bestimmt als Parameter für die Quantifizierung des Einflusses der Substitution auf die Nettoproteinsynthese. Die Substitution von 97,50% des K^+ durch Na^+ bewirkte einen signifikanten Rückgang der Proteingehalte. Da die Transpiration, das Wachstum und die Zuckerbereitstellung bei dieser Substitution nicht beeinträchtigt waren, stellte die Nettoproteinsynthese den empfindlichsten Prozess unter Substitutionsbedingungen dar.

Es konnte ausgeschlossen werden, dass ein durch die Substitution induzierter Magnesiummangel für die Hemmung der Proteinsynthese verantwortlich war. Auch ein Mangel an proteinogenen Aminosäuren konnte als limitierender Faktor ausgeschlossen werden. Es konnte sogar eine Akkumulation von freien Aminosäuren festgestellt werden. Daher wurde die Hypothese aufgestellt, dass der Prozess der Translation direkt durch die Substitution von K^+ durch Na^+ gestört wird.

Um diese Hypothese zu testen, wurden Ribosomen aus wachsenden Blättern von Zuckerrübe und Mais isoliert. Die Pflanzen wurden unter Kontrollbedingungen kultiviert. Die Translation durch Ribosomen aus Zuckerrübe und Mais wurde *in vitro* bestimmt über den Einbau von radioaktivem ^{35}S-Methionin in Peptide und Proteine. Um nur die Proteinsynthese cytosolischer Ribosomen zu erfassen, wurden die Ribosomen aus den Plastiden und Mitochondrien mit Chloramphenicol gehemmt. Zunächst wurden die Magnesium (Mg^{2+})- und K^+-Konzentrationen ermittelt, die den Anforderungen der Ribosomen beider Pflanzenarten entsprachen. Kalium wurde durch Na^+ substituiert (0%, 20%, 40%, 60%, und 80% Substitution). Der Einfluss der verschiedenen Substitutionsvarianten auf den Einbau von ^{35}S-Methionin wurde für die Ribosomen beider Pflanzenarten bestimmt.

Die *in vitro*-Translation beider Pflanzenarten reagierte empfindlich gegenüber der Substitution von K^+ durch Na^+. Dies zeigt, dass der Prozess der Translation direkt durch die Substitution gestört wird. Die *in vitro* beobachtete Störung der Translation erklärt die *in vivo* beobachtete Hemmung der Nettoproteinsynthese. Im Vergleich zu den Ribosomen aus Mais zeigten sich die Ribosomen der Zückerrübe toleranter gegenüber der Substitution von K^+ durch Na^+.

Neben der H^+-ATPase im Plasmalemma, stellen die Ribosomen das zweite Beispiel für eine Anpassung an hohe Na^+/K^+-Konzentrationsverhältnisse dar. Dies zeigt, dass die Salzresistenz von Halophyten nicht nur auf einer erfolgreichen Kompartimentierung beruht, sondern auch auf einer erhöhten Na^+-Toleranz von Schlüsselenzymen.

8 References

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9 Appendix

9.1 List of figures

Figure 1: Modified model of the plant response to salt stress according to Munns (1993), Schubert (2011), and Julkowska and Testerink (2015). Phase 0 includes short-term effects of salt stress on plant growth. Phase 1 is characterized by osmotic stress, while plants are affected by ion toxicity in Phase 2. The red line illustrates the response of a genotype which is sensitive in Phase 1 and 2. The green line shows a genotype which is partially resistant in Phase 1 and fully resistant in Phase 2.

Figure 2: Schematic illustration of *in vitro* approaches used to compare corresponding enzymes from halophytes and glycophytes.

9.2 List of abbreviations

ABA	abscisic acid
EC	electrical conductivity
ENA	exitus natrium-type Na$^+$-ATPase
ESP	exchangeable sodium percentage
FV	fast vacuolar
GA	gibberellic acid
HANA	*Heterosigma akashiwo* Na$^+$-ATPase
HKT	high-affinity potassium transporter
McSOS1	*Mesembryanthemum crystallinum SOS1*
NHX	Na$^+$/H$^+$-antiporter
NSCC	nonselective cation channel
PM	plasma membrane
ROS	reactive oxygen species

SOS1	Salt Overly Sensitive 1
SR	salt-resistant
SV	slow vacuolar

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Eidesstattliche Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

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Franziska Eva Faust