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Metabolic contribution to salt stress in two maize hybrids with contrasting resistance

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ABSTRACT

Salt stress reduces the growth of salt-sensitive plants such as maize. The cultivation of salt-resistant maize varieties might therefore help to reduce yield losses. For the elucidation of the underlying physiological and biochemical processes of a resistant hybrid, we used a gas chromatography mass spectrometry approach and analyzed five different salt stress levels. By comparing a salt-sensitive and a salt-resistant maize hybrid, we were able to identify an accumulation of sugars such as glucose, fructose, and sucrose in leaves as a salt-resistance adaption of the salt-sensitive hybrid. Although, both hybrids showed a strong decrease of the metabolite concentration in the tricarboxylic acid cycle. These decreases resulted in the same reduced catabolism for the salt-sensitive and even the salt-resistant maize hybrid. Surprisingly, the change of root metabolism was negligible under salt stress. Moreover, the salt-resistance mechanisms were the most effective at low salt-stress levels in the leaves of the salt-sensitive maize.

1. Introduction

Q2 Salt stress is a problem in many crops because it reduces growth [1] and therefore yield. With regard to increasing world food demand and the simultaneous prognosis of increasing soil salinization [2], attention should focused on improving salt resistance in important crop plants. Maize (*Zea mays* L.) is one of the main carbohydrate sources not only for human nutrition, but also for animal feed and bio-fuel production. Maize is furthermore considered a salt-sensitive crop plant [3]. It shows, on the one hand, a distinct reduction in leaf extensibility and reduced biomass production at salinity [4,5], resulting in enormous yield losses. On the other hand, the growth of the root system in which salt stress initially affects the plant appears to be less reduced than the growth of the maize shoot [6]. Mechanisms such as cell wall extensibility are partly responsible for such growth reduction and may be activated by the signaling from the roots to the shoot [7]. These findings underline the need to improve salt resistance in crop plants, especially in sensitive crops such as maize. In order to understand the integral mechanisms of the way that salt stress affects growth, we need to elucidate the biochemical and physiological reactions of maize under salt stress. In recent years, metabolite profiling has proved to be a powerful method, because it enables the analysis of a large set of compounds with high throughput [8,9]. Metabolomics extensively reflect phenotypic changes in certain tissues. These metabolite changes are induced by stress-related reactions based on altered gene expression [8], post-transcriptional protein modification [10], and changes in protein function. Some valuable metabolic datasets exist for several model and crop plants grown under saline conditions and show that various pathways are affected. In the model plant *Arabidopsis*, Kim et al. [11] have studied the metabolite changes in cell cultures after salt-stress treatment by means of a gas chromatographic–mass spectrometry/liquid chromatographic–mass spectrometry (GC–MS/LC–MS) approach and determined that the methylation cycle, the phenylpropanoid pathway for lignin production, and the glycinebetaine biosynthesis are the mainly affected metabolic pathways. In crop plants, biochemical targets during salt stress are amino acid synthesis, the tricarboxylic acid (TCA) cycle, sugar biosynthesis, and polyol synthesis [12–14]. These metabolome studies demonstrate that plant metabolites react strongly to salt stress.

However, a salt-sensitive plant species such as maize needs to get acclimated to salt stress during cultivation; otherwise, only shock reactions are detectable in metabolome studies. Therefore, a stepwise acclimation to hydroponic solution and to the salt stress is inevitable in our experimental design. Further investigations have shown that resistance adaptions are noticeable at the metabolic level in resistant varieties [13,14], but to date, no such data exist for salt-resistant maize. Amino acids and sugars serve as osmolytes, being widely accepted as resistance parameters indicating salt

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stress [15], but these findings needs to get proved for salt-resistant maize.

Roots show a different metabolic reaction from the leaves of plants under salt stress in detail [12–14]. The reason that such a large metabolic reaction takes place in leaves with no growth reduction in roots [16] remains unclear. To date, no specific approach for maize including an investigation of its leaves and roots and further including a salt-sensitive and a salt-resistant genotype exits. Dose-dependent metabolite changes, possibly with threshold and plateau behaviors as reported for *Lotus japonicus* under salt stress [15], are moreover often disregarded. With the aim of identifying salt resistance mechanisms, such behavior should be addressed; otherwise, an interesting resistance mechanism might remain undetected, because the wrong salt-stress level might have inadvertently been chosen. Therefore, we investigate to exposure maize hybrids to control conditions, minor moderate and high salt concentrations in five low-increasing salt increments. For maize, the physiology and the biochemistry of salt resistance are only rudimentarily understood. Our aim has been to close this gap in our knowledge by providing physiological and biochemical data concerning the resistance mechanisms in maize root and leaves based on a metabolome study. We have grown two hybrids differing strongly in salt resistance, *i.e.*, a salt-sensitive and a salt-resistant maize inbred line [16] under salt stress. The salt-sensitive hybrid Logo is a commercially cultivated hybrid (Limagrain, Chappes, France), which is used as double silage and corn type with no acknowledged resistance mechanisms to abiotic stress such as salt stress. As outcome of a screening for appropriate sensitive counterparts in former studies the used hybrid Logo showed a severe reaction to short term salt stress and therefore serves as a source for an appropriate salt-sensitive hybrid. The hybrid SR08 belongs to a closely related family of salt-resistant hybrids (SR), which was developed by crossing a distinct sodium-excluding inbred line (female) with different osmotic-resistant inbred lines (male) [16]. As a consequence the SR hybrids combine the two salt-resistance qualities Na^+ exclusion and osmotic robustness [16]. Na^+ exclusion which is inherited by one crossing parent, is especially a suitable quality for improving salt-resistance [17]. Moreover, the SR family is well characterized as showing marked differences in terms of growth reduction and hormonal responses under salinity as well as in growth promoting agents such as β-expansins in comparison to sensitive hybrids [5,7,18].

This metabolite study of two differently resistant maize hybrids might help to identify contrasting features with respect to metabolic alterations, thereby developing a clearer picture of the physiological and biochemical adjustments to salinity. New insights might help to provide useful tools for future breeding schemes or for the improvement of salt resistance by the screening of adequate physiological or biochemical characteristics. The GC/time-of-flight (TOF)-MS approach used here should enable us to answer following questions. (i) Which of the main pathways in maize leaves is affected during salt stress? (ii) Is root metabolism affected by long-term salt stress? (iii) Does the application of small salt-stress increments offer new insights into resistance mechanisms?

2. Materials and methods

2.1. Plant cultivation and processing

Two hybrids of *Z. mays* L. *cv.* Logo and *cv.* SR08 [16] were cultivated in a hydroponic solution (0.2 mM KH_2PO_4, 1 mM K_2SO_4, 2 mM $Ca(NO_3)_2$, 2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 150 μM Sequestrene® (Syngenta, Basel, Switzerland), 1 μM H_3BO_3, 2 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 5 nM $(NH_4)MoO$), and 1 g quarz sand (SiO_2) in a greenhouse. Five different salt-stress levels (0 mM (control),

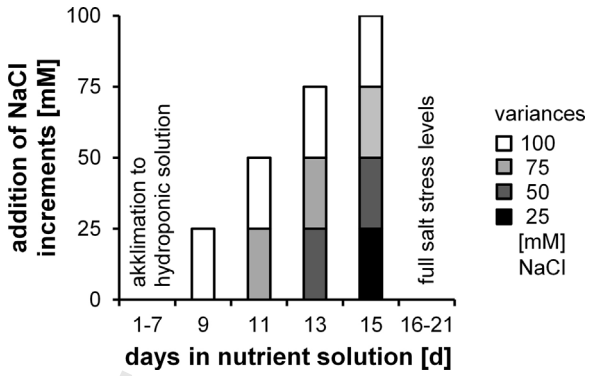

Fig. 1. Acclimation procedure to hydroponic solution and to salt stress. Hydroponic solution was increased in 25% increments every 2nd day and NaCl was added every 2nd day in 25 mM NaCl increments until the final concentration was reached. Plants were treated with 0 (control), 25, 50, 75, 100 mM NaCl, for 7d.

25 mM, 50 mM, 75 mM, and 100 mM NaCl) were imposed on each of four biological replicates ($n = 4$). Plants were adapted to nutrient solution in 25% increments every second day (Fig. 1). This course of action enabled the osmotic adaption of the plants to the full-strength nutrient solution. Afterwards, salt-stress concentrations were also increased in 25 mM increments every second day for osmotic adaption. Full stress treatment of 100 mM NaCl was achieved after 7 days and for lower salt stress levels even after a shorter akklimation period at the same time point (Fig. 1). Young shoots (*i.e.*, leaves that emerged after the achievement of 100% salt stress) and young root tips (*i.e.*, the first 3 cm) were harvested and immediately frozen in liquid nitrogen. All samples were homogenized by being ground with liquid nitrogen and were immediately freeze-dried. Additionally, the remaining root material was dried at 80 °C for the following Na^+ analysis.

2.2. Atomic absorption spectrometry and calculation of Na^+ uptake

For the cation analysis, ca. 50 mg freeze-dried leaf material and ca. 50 mg dried roots were weighed out into 10 mL 69% nitric acid. Subsequently, samples were solubilized by microwave digestion (leaves: 15 min, 190 °C, roots: 30 min, 190 °C). The solutions were diluted and filtered, and Na^+ analysis was carried out on an atomic absorption spectrometer (Thermo Fisher Scientific, 3300 series, Dreieich, Germany). Na^+ uptake was calculated as the ratio of total plant Na^+-content (root plus shoot) to total fresh weight (adapted from Schubert et al. [16]). Results were visualized by the use of Excel (Microsoft, Redmont, USA).

2.3. Metabolite extraction and metabolite profiling

Freeze-dried and homogenized sample material of roots and leaves, approximately 10 mg dry weight, were mixed with 360 mL pre-cooled methanol containing 0.02 mg/mL $^{13}C_6$-sorbitol (Sigma–Aldrich, Steinheim, Germany) as an internal standard for the correction of volume errors. Samples were extracted at 70 °C for 15 min. After a cooling step to room temperature, 200 μL $CHCl_3$ was added, and afterwards, the solution was agitated at 37 °C for 5 min. Finally, 400 μL H_2O was added in order to induce liquid phase separation. Samples were vortexed prior to centrifugation at 13,000 rpm for 5 min. A volume of 80 mL of the upper polar phase containing the primary metabolite fraction was dried in a vacuum concentrator (SpeedVac) overnight without heating and stored dry at −80 °C. Chemical derivatization, *i.e.*, methoxyamination and trimethylsilylation, and subsequent gas chromatography/time-of-flight-mass

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