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Title

Proteome analysis and pH sensitive ratio imaging: Tools to explore the decline in leaf growth under salinity

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Introduction

Soil salinity is one of the major environmental constraints limiting agricultural production worldwide. Irrigated land systems in arid and semiarid climates are especially adversely affected due to non-adapted irrigation practices. Current data indicates that more than 50% of all irrigated areas are already affected by soil salinity. In most saline environments NaCl is the predominant salt species, and principally adversely affects non-resistant plants by growth reduction due to an inhibition of cell division and cell elongation caused by osmotic effects, ion toxicity, and mineral disturbances in plants. However, the various deleterious effects of salinity on growth are not yet completely understood.

A first concept for understanding salt-induced growth reduction has been put forward by Munns (1993). The biphasic model of growth response explains that growth is reduced by osmotic stress in the first phase, while the second phase is dominated by ion toxicity. Since there is only small inter- and intraspecies variation in growth due to osmotic effects, sensitive and resistant genotypes can hardly be distinguished within the first phase but are distinguishable in the second phase. For the purpose of improving salt resistance to crops, the understanding of cellular mechanisms regarding reduced cell growth under saline conditions is essential.

Material and methods

The ratio imaging device used for this study was an inverse microscope (Leica DM IRB, Solms, Germany) connected to a high sensitive CCD-camera (CoolSNAP, Photometrics, Tucson, Arizona, USA) and coupled to a computer. Data acquisition and calculation of images was carried out with the Meta Fluor® imaging system (Visitron, Puchheim, Germany) using the program Meta Series (Vers. 6.2). Applying the dual excitation technique the adaxial side of the leaf was excited at 490 nm / 440 nm for pH by using the 20x objective (Leica pH 1; 20x / 0.40). Ratios were converted to pH values by in vivo calibration for pH (Mühling et al., 1995).

Plant proteins were isolated using an acetone-DTT based method and two dimensional (2D) polyacrylamide gel electrophoresis was carried out according to Zörb et al. (2004). Computer-assisted 2D analysis of each gel was done with Delta 2D software version 3.3 (Decodon, Greifswald). Protein spots were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Proteins were identified by searching the database based on MALDI TOF results of the peptide masses using the Mascot program package (<http://www.matrixscience.com>).

Result and Discussion

Effect of salt stress on leaf growth

Cell wall extensibility and turgor are two essential premises for plant growth (Hager, 2003). Although water potential and hydraulic conductivity are changed under saline conditions impeding water uptake by plants, turgor is not affected by salinity (Van Volkenburgh and Boyer 1985, Fan and Neumann 2004). Nevertheless, osmotic disturbances within the plant cell cause significant growth reduction during the first phase of salt stress. The repression of shoot growth in this phase is independent of genetic variations (Munns 1993) and occurs in all glycophytic plant species such as maize (*Zea mays* L.) or faba bean (*Vicia faba* minor). However, growth reduction was more pronounced in leaves of more sensitive genotypes (cv.

Pioneer 3906 and *Vicia faba*) compared to more resistant genotypes (SR03, Schubert and Zörb 2005) after 8 d salt treatment with 100 mM NaCl (Fig. 1).

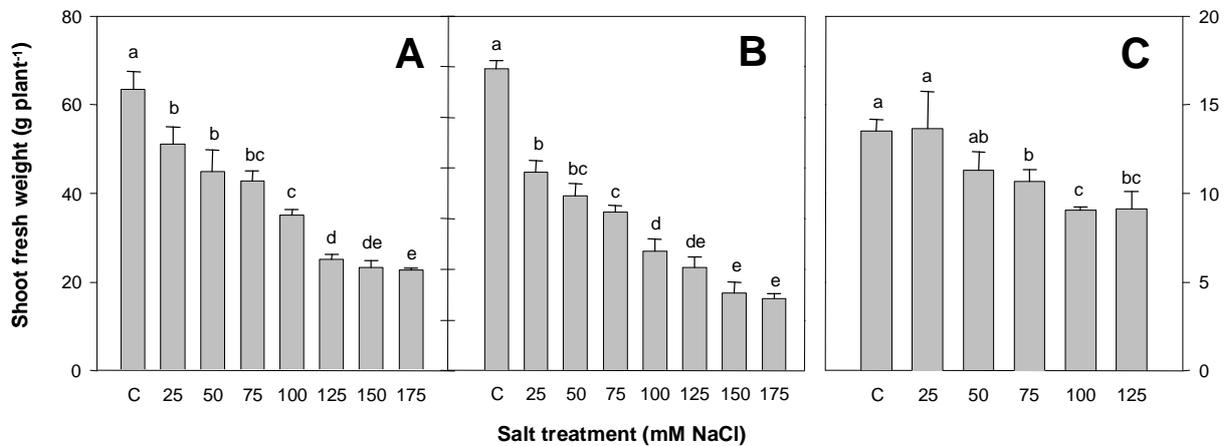


Figure 1. Shoot fresh weight of two maize genotypes, (A) SR03 and Pioneer 3906 (B), and *Vicia faba* (C) affected by treatment with 0-175 mM NaCl in nutrient solution. The values represent means \pm SE of four independent experiments. Significant differences ($P \leq 5\%$) are indicated by different letters. C, control plants without salt treatment (1 mM NaCl).

Effect of salt stress on apoplastic Na^+ concentration

Generally, increasing salt stress is accompanied by a linear increase of the Na^+ concentration both in roots and leaves (Fortmeier and Schubert, 1995). But depending on the resistance mechanisms plants such as maize are able to effectively exclude (Pioneer 3906) or include and exclude (SR03) Na^+ in contrast to *Vicia faba*. While root and shoot Na^+ is an adequate parameter to describe uptake and translocation, the apoplastic Na^+ concentration is still discussed to be an important trait in order to describe the decline in leaf growth (Mühling and Läuchli, 2002). Since intercellular K^+ concentration is directly affected by excess Na^+ , essential physiological parameters such as enzyme activity may be reduced.

While maize was able to maintain a non-toxic Na^+ concentration < 10 mM within the apoplastic space under saline conditions (Tab. 1), *Vicia faba* showed a dramatic increase of the Na^+ concentration. Although, the Oertli-hypothesis (Oertli 1968) states that a high apoplastic Na^+ concentration affects plant growth, which could not be confirmed particular for maize, it cannot be excluded that concentrations of > 50 mM Na^+ play a role in salt-induced growth reduction in *Vicia faba* (Tab. 1).

Table 1. Effect of salt stress on K^+ and Na^+ concentration (mM), and K^+/Na^+ ratio in apoplastic fluid of maize cv. Pioneer 3906 and *Vicia faba*. The values represent means \pm SE of four independent experiments. Significant differences ($P \leq 5\%$) are indicated by different letters.

Salt treatment	K^+ (mM)	Na^+ (mM)	K^+/Na^+ ratio
Pioneer 3906			
Control	35.4 \pm 4.9 a	0.85 \pm 0.06 a	51.6 a
100 mM NaCl	27.5 \pm 4.2 a	7.07 \pm 0.57 b	4.7 b
<i>Vicia faba</i>			
Control	3.3 \pm 0.24 b	0.63 \pm 0.29 a	5.2 b
100 mM NaCl	14.6 \pm 2.3 c	49.5 \pm 8.33 c	0.29 c

Salt-induced changes of apoplastic pH

According to acid-growth considerations (Hager 2003), acidification of the leaf apoplast mediated by the plasma membrane H^+ -ATPase is a major premise to increase cell wall extensibility. To investigate changes of apoplastic pH as affected by salt stress and its role in growth reduction, ratio imaging by pH-sensitive fluorescent dyes is an adequate technique for *in vivo* measurements (Pitann et al. 2009a).

Using FITC-dextran (50 μ M) as a suitable fluorescent dye, a significant apoplastic alkalization of the salt-sensitive Pioneer 3906 and *Vicia faba* could be shown, whereas this was not valid for the more resistant SR03 (Fig. 2). This is in line with findings of Felle and Hanstein (2002) which confirm an apoplastic alkalization in bean in the same pH range under stress-conditions. Likewise, instant growth activation by lowering the apoplastic pH was found for maize (Peters et al. 1998). Thus an inversion of the acid-growth argument would permit the conclusion that in growing tissues a pH increase of less than half a unit is sufficient to inhibit growth.

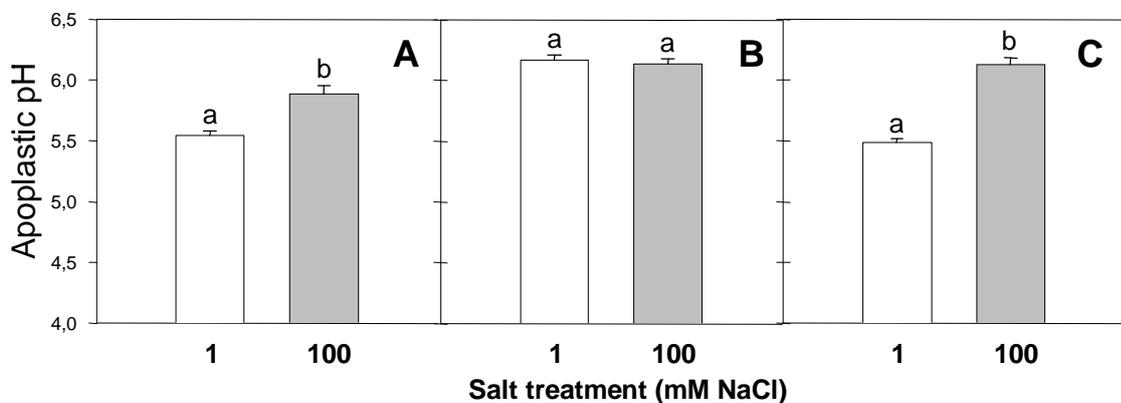


Figure 2. Effect of salt stress on apoplastic pH in intact leaves of two maize hybrids and *Vicia faba* analysed by ratiometric fluorescent microscopy using fluorescein isothiocyanate-dextran (FITC, 50 μ M) at excitation wavelengths of 490 and 440 nm: (A) Pioneer 3906; (B) SR03; (C) *Vicia faba*. The values represent means \pm SE of four independent experiments. Significant differences ($P \leq 5\%$) are indicated by different letters.

Proteome analysis

The adaptation to salt stress has a strong influence on the proteome (Zörb et al. 2004). By identifying differentially regulated proteins using two dimensional (2D) polyacrylamide gel electrophoresis combined with MALDI-TOF mass spectrometry, plenty of diverse proteins which are involved in the physiology of salt resistance were detected. The use of 2D proteomics enables the analysis of hundreds of proteins simultaneously by comparing protein concentrations in treated and non treated plants.

The biochemical reaction of maize to salt stress was characterized by a mitigation of symptoms and not by a specific adaptation (Zörb et al., 2004). In this study, the proteome was analysed after a long term salt treatment with 100 mM NaCl showing that there was an uncontrolled change of 64% of the leaf proteins. Mild stress (25 mM NaCl) still leads to the change of one third of the detected proteins. Both conditions appear to cause a non-specific adaptation to salt stress in maize at the level of proteins rather than a mitigation of common stress symptoms. Plants which were adapted to long term salt stress show different responses which were triggered by osmotic stress as well as ion toxicity resulting in a massive change of

the protein pattern. We therefore focus on the effects of the analysis of apoplastic proteins using the two-dimensional technique. The role of apoplastic proteins in growth regulation after salt stress would be helpful to understand the mechanism of salt stress related growth reduction.

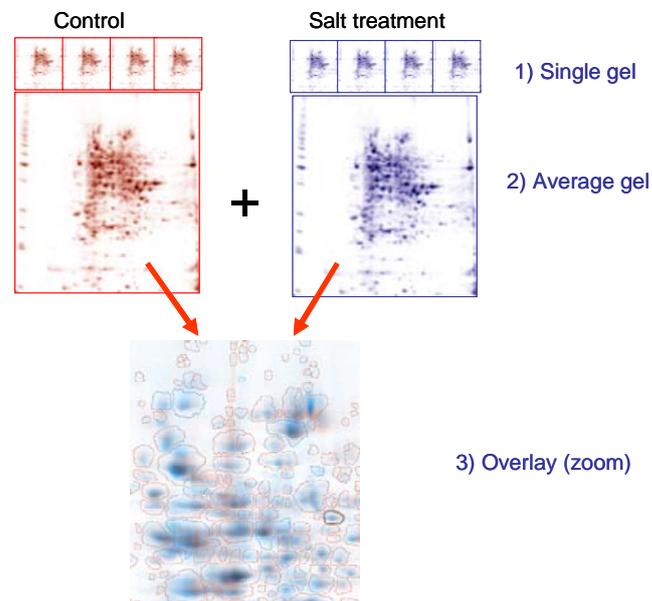


Figure 4. 1) Two 2D-gels showing total protein extract from maize leaves, 2) average gel and 3) overlay of salt treatment versus control.

Expansin expression

For salinity inhibited growth aspects, apoplastic proteins such as expansins were of great interest. Expansins are wall-loosening enzymes, located within the apoplast of the elongation zone of leaves (Cosgrove, 2000). They are known to be acid-activated and have the unique property of cell wall-loosening below pH 5 (Cosgrove, 2000; (Fig. 5). An apoplastic acidification leading to a pH range within the expansins pH-optimum is the major requirement to increase cell wall extensibility, which controls extension growth. Because under salinity the apoplastic pH is increased leading to an alkalization (Pitann *et al.*, 2009b), a reduced activity of expansins may occur causing a limited shoot growth. We use the 2D proteomics approach to show the effect of salinity to the relative concentration of a maize expansin protein.

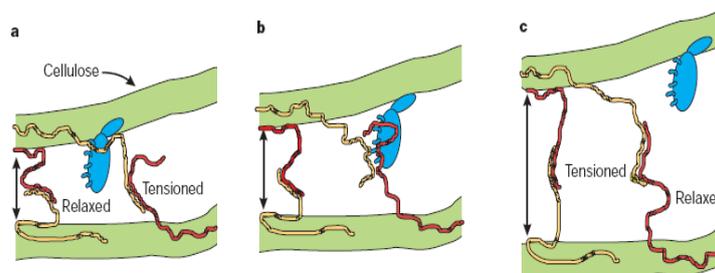


Figure 5. Mechanism of cell wall loosening by expansins, cellulose microfibrills (green ribbons), glykans (red and yellow ribbons). Expansins (blue) loosening H-bonds of glykans and cellulose microfibrills (a) or between the glykans (b) reversible, tensioned or relaxed. (c) The expansion of the polymer structures stops as the relaxed glycan chain tensioned (according to Cosgrove 2000).

We demonstrate that the activity of expansins was reduced under salt stress especially in the less resistant maize hybrid Pioneer 3906 (Pitann et al., 2009c). A β -expansin protein concentrations (Acc. No. gi|14193763, Fig. 6A) dramatically decreased by 63% in comparison to the control plant. The decrease of the β -expansin may trigger the lower leaf extension growth and the lower biomass production, especially in the salt sensitive genotype. Additionally, the changes in expansin transcript abundance of maize (β -expansin mRNA) were analyzed. We examined the transcript level of β -expansin 3 (*ZmEXPB3*) by the use of quantitative real-time PCR. According to the protein abundance, *ZmEXPB3* transcript level of the salt-sensitive maize genotype Lector decreased under salinity by 70% in comparison to the corresponding control, whereas the resistant hybrid SR03 showed an increment of the transcript level by 200% (Fig. 6B).

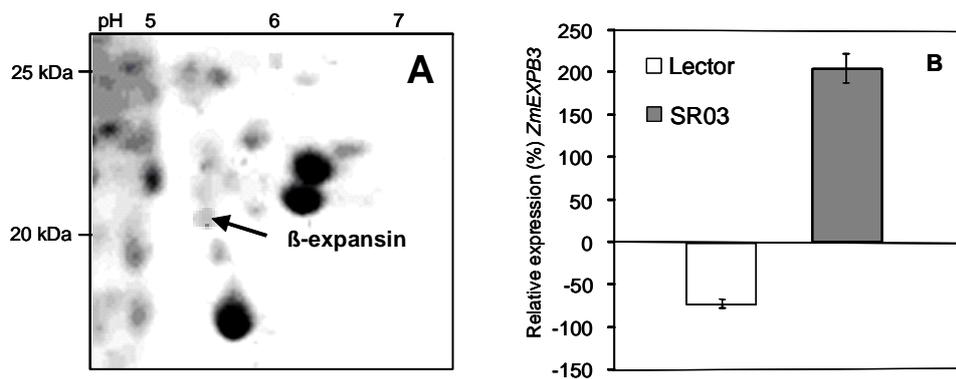


Figure 6

(A) Representative 2D gel image zoom of the maize hybrid Pioneer 3906. Zoom region of the 2D gel with β -expansin 2, as indicated by an arrow.

(B) Expression profile of an β -expansin transcript (*ZmEXPB3* mRNA) analyzed by quantitative real-time PCR. Salt-sensitive maize hybrid Lector, salt-resistant hybrid SR03, 100 mM NaCl treatment. Control plants (1 mM NaCl) were harvested at the same age. cDNAs extracted from leaves were used as templates for real-time PCR. The relative expression intensity was calculated according to $2^{-(\Delta\Delta C_t)}$ (*ZmEXPB3* - $\Delta\Delta C_t$ ubiquitin conjugating enzyme). Values are means of three replicates \pm SE.

In conclusion, the transcript level and the proteome show a higher level of β -expansin in resistant maize cultivars compared to more sensitive cultivars, which may contribute to better growth and improved salt resistance.

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