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Assessing the importance of the root system in long-distance versus local phytohormone signalling

Introduction

Salinity limits plant productivity by modifying plant hormonal and nutritional balance, while root growth maintenance is considered to be an adaptive response. Abscisic acid (ABA) and cytokinins (CKs) are phytohormones that mediate plant response to abiotic stress and are mainly considered to be produced in the roots. Most studies devoted to the role of phytohormones in stress physiology have focused on the stress hormones such as ABA and ethylene and ignored other possible interactions. ABA and CKs have long been considered antagonists but the role of this antagonism in mediating abiotic stress resistance has received scant attention.

Despite much research on root-to-shoot signalling, it is uncertain whether shoot hormonal changes following salt stress are due to changes in root hormone export, or local metabolic changes in the shoot. The conflicting data about the role of root hormone supply and its physiological impact on the shoot suggests the role of root-synthesised CKs and ABA in regulating shoot responses requires re-evaluation. To better understand this process, the target of the STSM was to elucidate CKs-ABA interaction in shoot growth under salinity.

Materials and methods

Plant material

For this purpose, a functional experimental approach modified both CKs and ABA concentrations in the domestic tomato. Two independent sets of tomato plants (*Solanum lycopersicum* L.) were used. The first set of plants overexpressed *NCED* genes encoding enzymes involved in ABA biosynthesis constitutively under the control of the Gelvin superpromoter. The transgenic line is termed sp12 and its wild type (WT) is the line Ailsa Craig (AC). The second set of transgenic plants used overexpressed *IPT* genes that encode enzymes involved in CKs biosynthesis constitutively under the control of the CaMV 35S promoter (*35S::ipt*). The transgenic line used is termed iptG and its WT is the line UC82B.

Plant culture

Seeds were sown in commercial vermiculite depending on their germination performance, watered with deionized water and kept at 26-28ºC, 80-90% relative humidity and darkness. Since germination times differed between genotypes, a schedule of sowing was established to synchronise growth of the NCED line with the rest of the genotypes. Thus sp12 seeds were sown one week before all other lines. Five or seven days after sowing each genotype, growth chamber conditions were settled to 16-h day (photon flux density was 245 µmoles m^{-2} s^{-1}) and 8-h night cycle with a day/night temperature of 18/25ºC and 40-60% relative humidity and the seedlings were daily watered with half-strength Hoagland solution. Relative humidity was maintained at 40-60%.

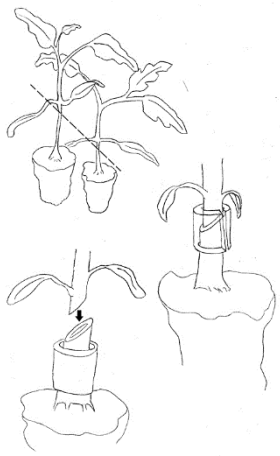

Figure 1 – Grafting technique using the splicing method

Grafting allows the role of long-distance CK and ABA signalling in mediating shoot physiological responses and hormonal concentrations to be assessed. Grafting was performed using the splicing method at the two to three true leaf stages (3–4 weeks after sowing), as is common commercially, and the *scion* was attached at the first node of the rootstock.

Reciprocal grafting between different transformants resulted in 16 scion/rootstock combinations: AC/AC, AC/sp12, sp12/AC, sp12/sp12, UC82B/UC82B, UC82B/iptG, iptG/UC82B, iptG/iptG, AC/UC82B, UC82B/AC, AC/iptG, iptG/AC, UC82B/sp12, sp12/UC82B, sp12/iptG and iptG/sp12. After grafting, plants were kept for one week in darkness and 100% RH. When grafts were established, grafted plants were adapted eventually, within a period of one week, to the cabinet conditions described above.

Once plants were adapted, they were transferred to a hydroponic culture system and fixed on 16 L plastic black containers floating on aerated half-strength modified Hoagland solution. A factorial design of 16 graft combinations x 2-salt treatments x 5 replicates was performed. The five replicates were randomly distributed in the containers. Furthermore, containers were randomly distributed in the growth chamber. After one week of acclimatization to the hydroponic system, the plants were exposed to 0 (control treatment) or 100mM of NaCl (salt treatment) added to the nutrient solution for 21 days. In both salt and control treatments, the nutrient solution was refilled daily and replaced twice every week. The pH was adjusted to 5.6-5.8. Final electric conductivity (EC) of the salinity-amended Hoagland solution ranged 9-10 mS cm^{-1}.

Plant measurements

Vegetative growth (leaf area, shoot and root fresh weight) was assessed at the end of the experiment (21DAT). Leaf area was determined using an area meter (model LI-3100C; LI-Cor, Lincoln, NE, USA). Shoot and roots were separated immediately and weighed for biomass determination. Young fully expanded leaves and young roots were immediately frozen in liquid nitrogen and stored at -80ºC for further hormonal analysis.

Once plants were de-topped at the graft union, the root system was placed inside a Scholander pressure vessel (Soil Moisture Equipment Corp., Model 3000F01, Santa Barbara, CA, USA), in order to collect root xylem sap. At certain pressures (0.2 - 0.8 MPa, depending on the plant genotype), sap was collected during 3 – 4 minutes, and placed in a pre-weighed Eppendorff tube, immediately frozen with liquid nitrogen and stored at -80 °C for further hormonal analysis.

Throughout the experiment, photosynthesis (A) and gas exchange parameters (g_s) were monitored using a CIRAS-2 (2PP System, Massachusetts, USA) starting at 9.00am. CO_2 was set at ambient levels (400 ppm) and radiation was matched to the chamber conditions (1000 µmol m^{-2} s^{-1} PFPD).

Results

Shoot FW

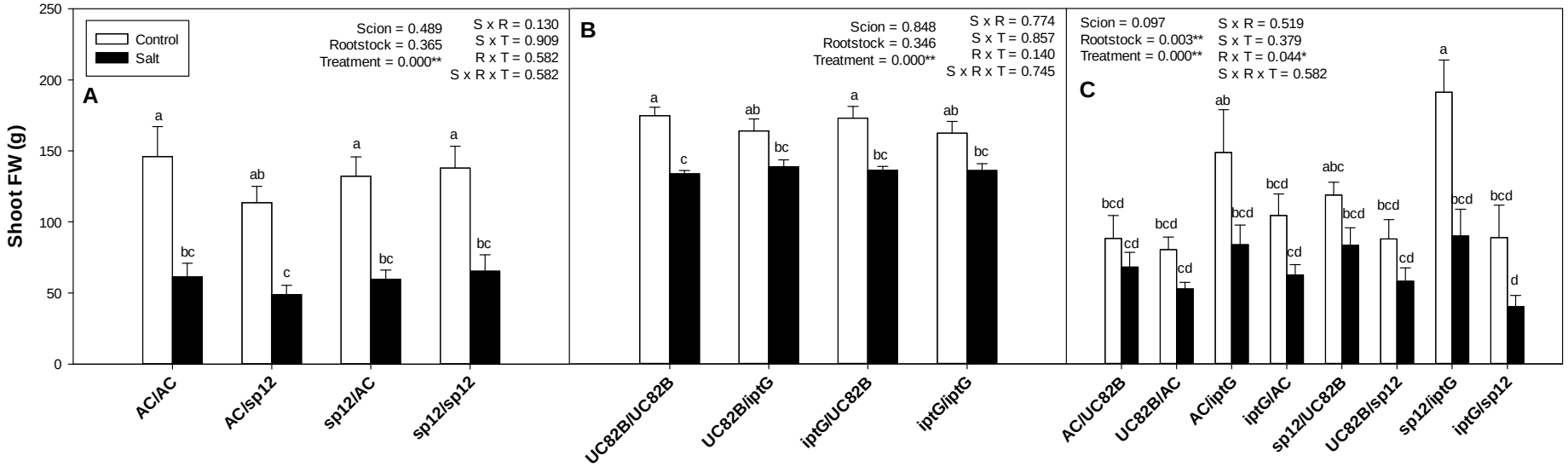

Figure 2: Final shoot FW 16 graft combinations after 21 days of 0 and 100mM NaCl treatment. Transgenic line sp12 and its WT-AC (A), transgenic line iptG and its WT-UC82B (B), and reciprocal grafting among *NCED* and *IPT* lines (C). Data are means ± SE of 5 replicates. Results of three-way ANOVA (P Values reported) are indicated within each panel. Letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

The different graft combinations between AC and sp12 showed no differences in shoot FW under control or salt conditions (Figure 2A). Salinity decreased shoot growth in 54% similarly in the four combinations. Grafting combinations between UC82B and the transgenic line iptG (Figure 2B) were more vigorous (37% higher shoot FW) than the combinations AC-sp12 (Figure 2A). The different graft combinations between UC82B and the transgenic line iptG showed no differences in shoot FW under control or salt conditions (Figure 2B). Salinity decreased shoot growth by around 15% in all combinations equally. Reciprocal grafting among *NCED* and *IPT* lines (Figure 2C) revealed a significant rootstock effect on shoot growth (3-way ANOVA within the panel), with the iptG transgenic rootstock (AC/iptG and sp12/iptG) increasing shoot FW by 45% (compared to all other graft combinations) under control conditions. Although salinity decreased shoot growth of all graft combinations (by an average of 41% across all graft combinations), there was a significant ($P<0.05$) rootstock x treatment interaction. Although all graft combinations showed a similar shoot FW when salinized, graft combinations with iptG rootstocks showed greater growth inhibition (45% and 53% for AC/iptG and AC/sp12 respectively) compared to the other graft combinations.

Root growth of the four different AC-sp12 graft combinations and the four different UC82B-iptG graft combinations was insensitive to salinity (no significant treatment effect – Fig 3A, B). The UC82B-iptG graft combinations were more vigorous than the AC-sp12 ones (Figure 3B), similar to the effect on shoot FW. When *NCED* lines were reciprocally grafted with *IPT* lines (Figure 3C) rootstock had a significant ($P<0.05$) impact on root FW (3-way ANOVA within the panel). Irrespective of salinity treatment, graft combinations with the iptG rootstock had a higher root FW than those with the sp12 rootstock.

For all graft combinations, total FW values (Figure 4) showed similar responses to shoot FW (Figure 2), which is not surprising as shoot FW comprised 78% of the total biomass (averaged across all graft combinations). In the same way, similar results were found for leaf area.

Root FW

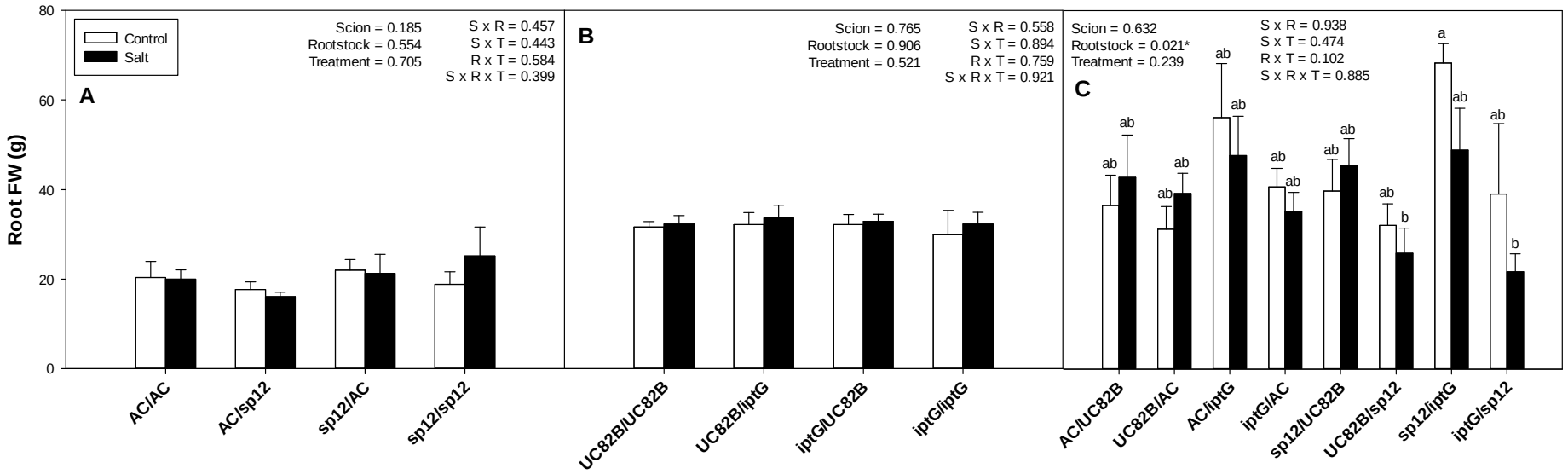

Figure 3: Final root FW of the 16 graft combinations after 21 days of 0 and 100mM NaCl treatment. Transgenic line sp12 and its WT-AC (A), transgenic line iptG and its WT-UC82B (B), and reciprocal grafting among *NCED* and *IPT* lines (C). Data are means ± SE of 5 replicates. Results of three-way ANOVA (P Values reported) are indicated within each panel. Letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Total FW

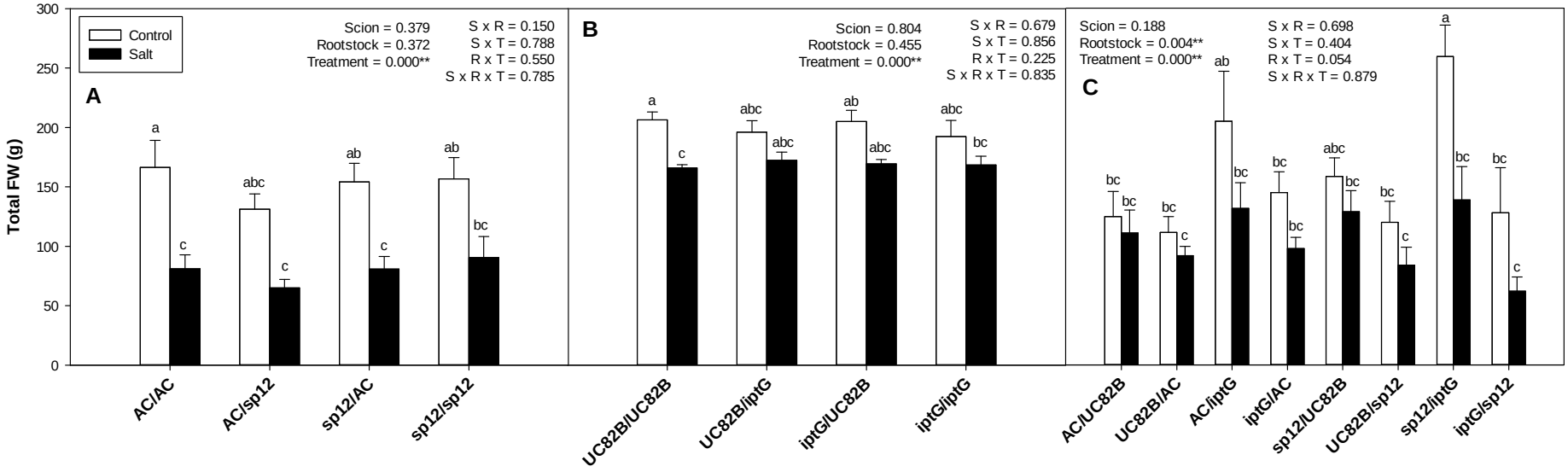

Figure 4: Final total FW of the 16 graft combinations after 21 days of 0 and 100mM NaCl treatment. Transgenic line sp12 and its WT-AC (A), transgenic line iptG and its WT-UC82B (B), and reciprocal grafting among *NCED* and *IPT* lines (C). Data are means ± SE of 5 replicates. Results of three-way ANOVA (P Values reported) are indicated within each panel. Letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Leaf area

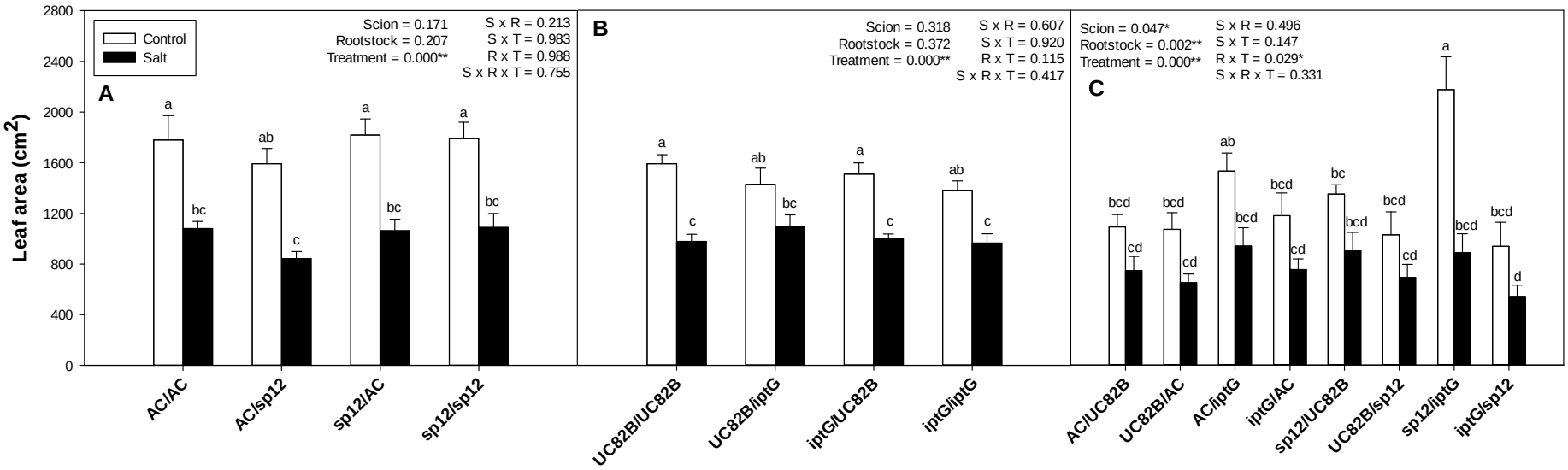

Figure 5: Final leaf area of the 16 graft combinations after 21 days of 0 and 100mM NaCl treatment. Transgenic line sp12 and its WT-AC (A), transgenic line iptG and its WT-UC82B (B), and reciprocal grafting among *NCED* and *IPT* lines (C). Data are means ± SE of 5 replicates. Results of three-way ANOVA (P Values reported) are indicated within each panel. Letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

After 15 days under control conditions, A was similar for self- and reciprocal grafts between AC and sp12 (Figure 6A). Salinity tended to decrease A (P=0.065) in all graft combinations, except in sp12/AC where it was maintained. Under control conditions, the UC82B-iptG combinations (Figure 6B) showed no differences in A, but A levels were 16% higher than in AC-sp12 combinations (Figure 6A). Salinity decreased A similarly in the four UC82B-iptG combinations, but only significantly in UC82B/UC82B (19%) and iptG/UC82B (36%) (Figure 6B). Under control conditions, A of AC/iptG was higher than the rest of the genotypes, but significantly with UC82B/AC and sp12/UC82B (Figure 6C). The salinity treatment applied decreased A in almost all graft combinations, except in sp12/UC82B where A was surprisingly higher (50% than its respective control). Under salinity, A was significantly higher when IPT lines (genotypes UC82B and iptG) were used as rootstocks, compared with the combinations whose roots were AC, or especially sp12 (Figure 6C).

The presence of the transgenic line sp12 as a rootstock, scion, or self-grafted didn't decrease significantly gas exchange (g_s) compared with the WT-AC self-grafted under control conditions (Figure 7A). Salinity decreased g_s in all AC-sp12 grafting combinations between 60 and 75%, except in sp12/AC where this reduction was lower than 20% (Figure 7A). UC82B-iptG combinations showed 38% higher g_s (Figure7B) than the AC-sp12 ones (Figure 7A). Under control conditions iptG/UC82B was the only combination that showed significant lower (39%) than the other 3 (Figure 7B). After 15 days of salinity treatment, g_s was reduced significantly in all graft combinations, ($P<0.001$), but more so in UC82B/UC82B and iptG/iptG plants, where the decrease was greater than 50% compared with the control (Figure 7B). When the *NCED* and *IPT* lines were reciprocally grafted (Figure 7C), transgenic lines sp12 and iptG used as a rootstocks had higher g_s than the other two lines. Salinity significantly decreased g_s in almost all graft combinations by around 80%, except when the rootstock was UC82B (AC/UC82B and sp12/UC82B), where this decrease was just around 15% and not statistically significant.

Reciprocal grafting AC-sp12 (Figure 8A) showed no significant differences between genotype combinations in terms of WUEi under control conditions. Salinity increased WUEi in all genotypes (Figure 8A), 48% on average, but only significantly in sp12 self-grafted. Reciprocal grafting between UC82B and iptG (Figure 8B) showed 34% lower WUEi than AC-sp12 combinations (Figure 8A). Under control conditions, the combination iptG/UC82B had higher WUEi than the other 3 (Figure 8B). The application of salt treatment for 15 days increased WUEi in 46% across genotypes ($P<0.001$), but this was not significant in ipt/UC82B (Figure 8B). Reciprocal grafting between *NCED* and *IPT* lines (Figure 8C) exhibit not significant differences in WUEi under control conditions for the 8 combinations. Salinity increased WUEi significantly only in AC/iptG (60%) and in sp12/UC82B (43%) (Figure 8C).

Photosynthesis

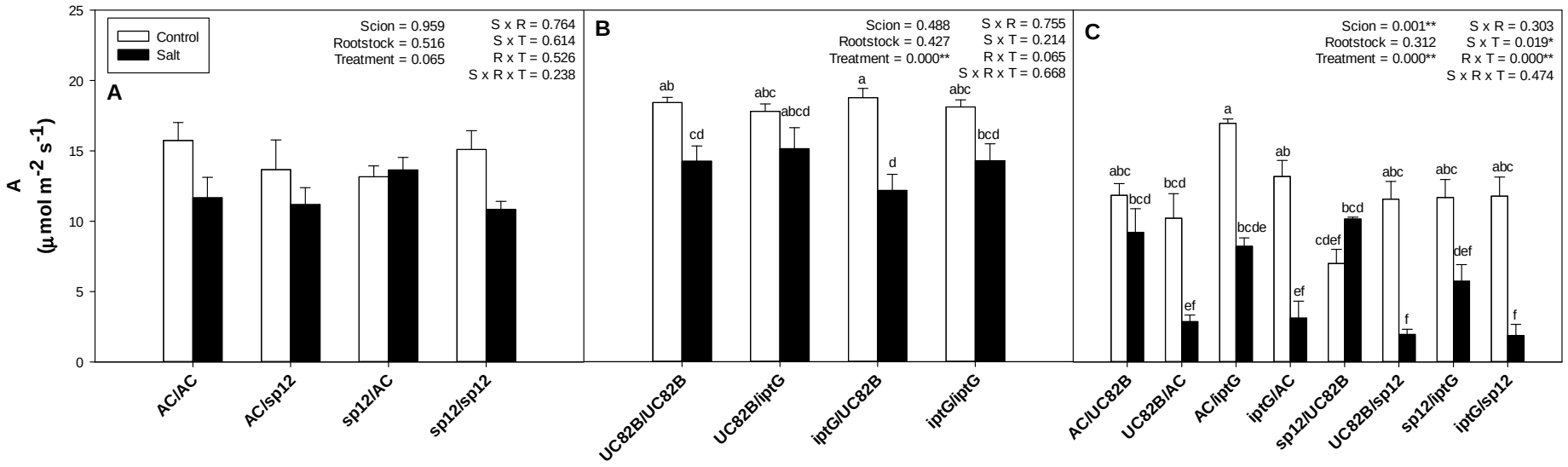

Figure 6: Net photosynthetic rate of the 16 graft combinations after 15 days of 0 and 100mM NaCl treatment. Transgenic line sp12 and its WT-AC (A), transgenic line iptG and its WT-UC82B (B), and reciprocal grafting among *NCED* and *IPT* lines (C). Data are means ± SE of 5 replicates. Results of three-way ANOVA (P Values reported) are indicated within each panel. Letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Stomatal conductance

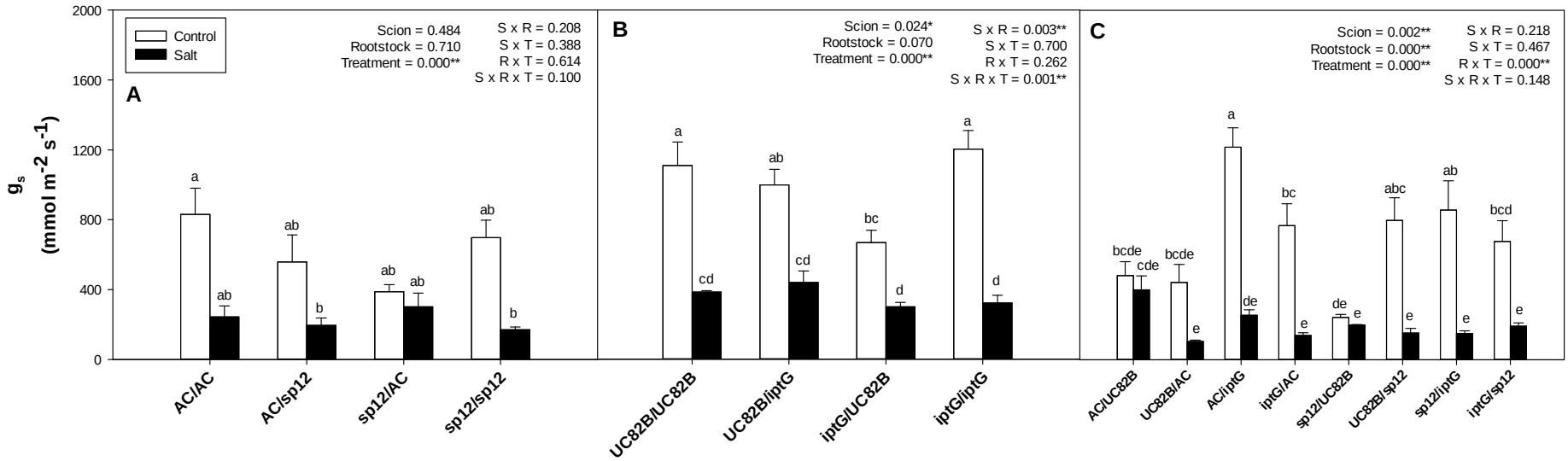

Figure 7: Stomatal conductance of the 16 graft combinations after 15 days of 0 and 100mM NaCl treatment. Transgenic line sp12 and its WT-AC (A), transgenic line iptG and its WT-UC82B (B), and reciprocal grafting among *NCED* and *IPT* lines (C). Data are means ± SE of 5 replicates. Results of three-way ANOVA (P Values reported) are indicated within each panel. Letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

WUEi

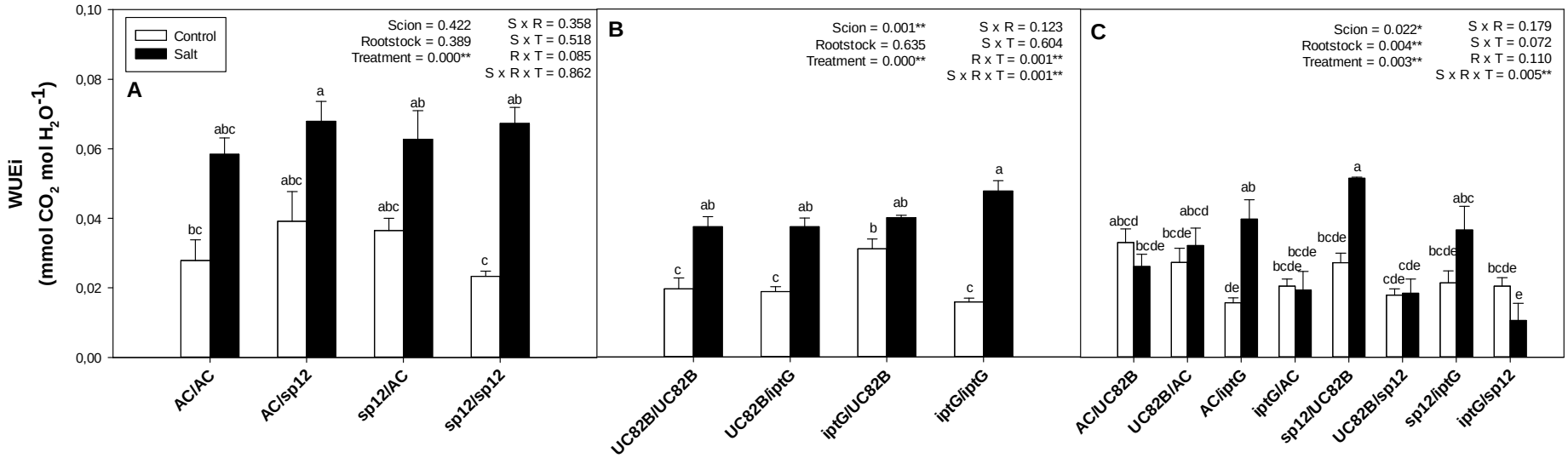

Figure 8: Water use efficiency (WUEi = A/g_s) of the 16 graft combinations after 15 days of 0 and 100mM NaCl treatment. Transgenic line sp12 and its WT-AC (A), transgenic line iptG and its WT-UC82B (B), and reciprocal grafting among *NCED* and *IPT* lines (C). Data are means ± SE of 5 replicates. Results of three-way ANOVA (P Values reported) are indicated within each panel. Letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Reciprocal grafting of the AC and sp12 lines resulted in no significant differences between graft combinations or salt treatments (Figure 9A).Under control conditions, there were no significant differences in root xylem ABA concentration among graft combinations of the UC82B and iptG lines (Figure 9B), but their ABA levels were on average 75% lower than in the AC-sp12 graft combinations (Figure 9A). Salinity significantly increased ABA concentration in UC82B/iptG, iptG/UC82B and iptG/iptG (5-fold concentration compared with the control), and not significantly in UC82B/UC82B (3.2-fold the control levels - Figure 9B). Under control conditions, reciprocal grafting between *NCED* and *IPT* lines generally decreased ABA concentration compared with AC-sp12 (Figure 9A) and UC82B-iptG combinations (Figure 9B), and resulted in no significant differences in ABA concentration among the different graft combinations (Figure 9C). Only using the transgenic line sp12 (UC82B/sp12 and iptG/sp12) as a rootstock seemed to increase root xylem ABA concentration (Figure 9C), but this increase wasn't statistically significant. Salinity increased ABA concentrations in all graft combinations, except in AC/UC82B where it was maintained (Figure 9C), but only significantly when the ABA-overproducing transgenic line sp12 was used as rootstock (UC82B/sp12 and iptG/sp12), as indicated by a significant rootstock x treatment interaction. Thus the sp12 rootstock increased root xylem ABA concentration (but not when grafted to its wild-type).

Under control conditions, AC-sp12 combinations (Figure 10A) showed no significant differences in root xylem CKs concentration, although these CKs levels were 62% lower in sp12/AC compared to other graft combinations. Generally, salinity increased CKs concentration in the four graft combinations, but especially in sp12/AC (5-fold higher than control conditions). Surprisingly, under control conditions, root xylem CKs concentration did not differ in the 4 UC82B-iptG combinations (Figure 10B). Salinity increased root xylem CKs concentration 11-fold on average. The presence of the CKs-overproducing iptG transgenic line in the grafting combinations, as a scion, as a rootstock, and highly when this line was self-grafted, enhanced the CKs concentration, pointing to an effect of the genotype on the greater presence of these hormones (3-way ANOVA within the panel). Figure 10C reveals that there wasn't a clear effect of the reciprocal grafting between *NCED* and *IPT* lines on CKs concentration under control or salinity conditions. The trend was that the concentration of these hormones was higher when UC82B or sp12 were used as rootstocks, and lower when the rootstock was AC, but results were very uncertain.

ABA root xylem sap

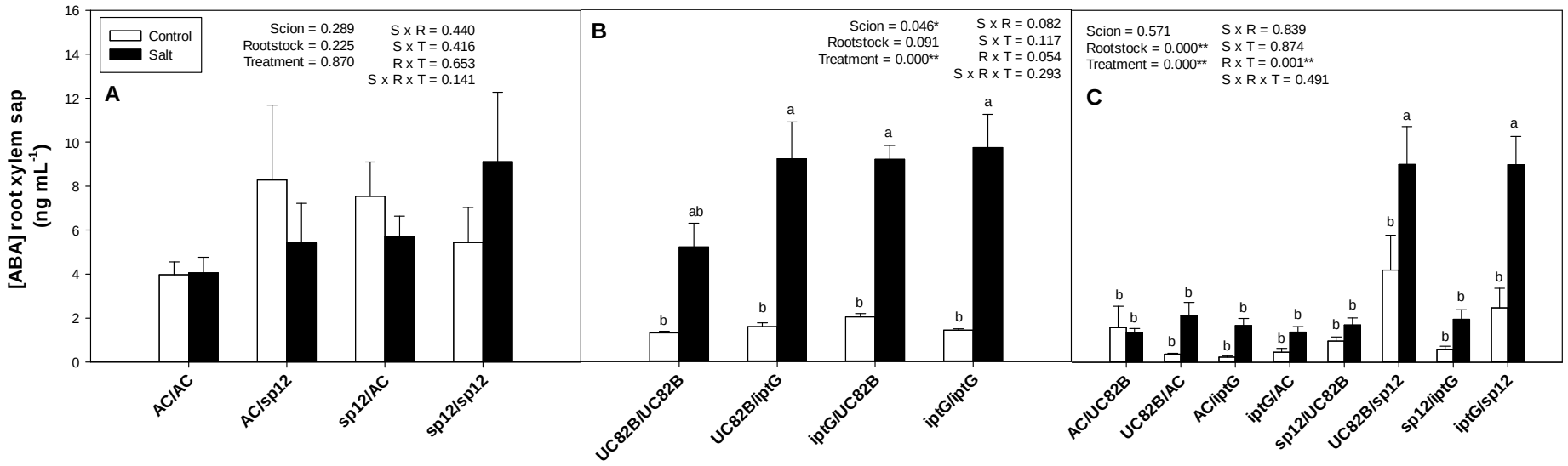

Figure 9: ABA concentration in the root xylem sap of the 16 graft combinations after 21 days of 0 and 100mM NaCl treatment. Transgenic line sp12 and its WT-AC (A), transgenic line iptG and its WT-UC82B (B), and reciprocal grafting among *NCED* and *IPT* lines (C). Data are means ± SE of 5 replicates. Results of three-way ANOVA (P Values reported) are indicated within each panel. Letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Cks root xylem sap

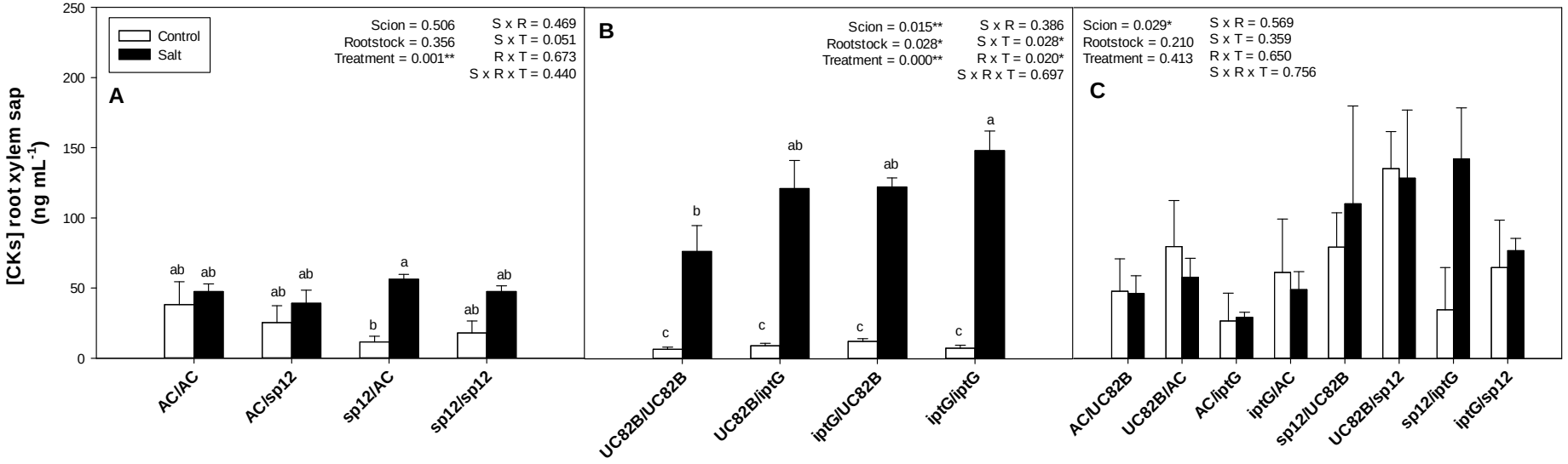

Figure 10: CKs concentration in the root xylem sap of the 16 graft combinations after 21 days of 0 and 100mM NaCl treatment. Transgenic line sp12 and its WT-AC (A), transgenic line iptG and its WT-UC82B (B), and reciprocal grafting among *NCED* and *IPT* lines (C). Data are means ± SE of 5 replicates. Results of three-way ANOVA (P Values reported) are indicated within each panel. Letters above bars indicate significant differences as determined by Tukey pair-wise analysis.

Discussion

As it has been seen in previous experiments (data not shown) using this culture system, the differences in terms of plant growth between salinity treatments or among genotypes are due to differences mainly in the shoot. Differences in root growth were attenuated when this hydroponic culture was used.

Surprisingly, differences in growth among AC-sp12 combinations under control conditions were not found, in contrast with other works that reveal lower biomass of the transgenic line sp12 compared with its WT-AC grown as a whole plant hydroponically (Thompson et al, 2007a) or reciprocal grafting between ABA-deficient mutant *flacca* and the same WT-AC (Chen et al, 2010). Similar grafting combinations (Thompson et al, 2007b) showed a decreased in the photosynthetic rate and in the stomatal conductance when the transgenic line sp12 was used as *scion*, along with an increase in ABA concentration in the leaf but not in the root xylem sap. In our results, the presence of sp12 in the shoot had less impact, especially on the stomatal conductance. Salinity decreased growth in the four combinations equally, which was correlated with a decrease in stomatal conductance, but not with the ABA levels found in the xylem sap.

Since the genetic background of the transgenic line iptG and its WT-UC82B was different, the behaviour of these two lines grafted was different from the previous ones under the same conditions. Plants resulted from the reciprocal grafting between UC82B and iptG seemed to be more vigorous than the AC-sp12 combinations. The presence of the transgenic line iptG in the root overexpressing the *IPT* gene didn't have any effect on plant growth under control conditions. However, according to bibliography, under 100mM NaCl treatment for 21 days, IPT-expressing rootstocks improved shoot FW 2-fold WT rootstocks (Ghanem et al, 2011), which was not found on these results. Another result that contrast with the bibliography is the increase of the CKs concentration in the root xylem sap when the salinity treatment is applied. Yang et al (2002) found that the water stress decreased Zeatin + Zeatin riboside concentration in root exudates in rice plants. However, Ghanem et al (2011) found that when these *IPT*-overexpressing lines were used as rootstocks, the CKs levels in the root xylem sap were higher compared with the WT. These increased CKs levels could explain the low level of growth decrease of these UC82B-iptG combinations under salinity (Ghanem et al, 2011).

The reciprocal grafting between NCED and IPT lines revealed the most confusing results. Under control conditions, plant FW decreased compared with AC-sp12 and UC82B-iptG combinations, which suggests a possible negative interaction when grafting these two set of plants. Under salinity, plant growth of the different eight combinations can be more explained in terms of genetic background of the rootstock than in terms of photosynthesis, gas exchange or hormones.

As a final conclusion, hormones in the leaf should be analysed in order to know further if these results can be better explained in terms of ABA or CKs concentration.

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