Title: Analysis of salt-induced physiological and proline changes in 46 switchgrass (*Panicum virgatum*) lines indicates multiple response modes

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Short Running Title: Salt Stress Tolerance Assessment of Switchgrass

ABSTRACT

Switchgrass (*Panicum virgatum*) is targeted as a biofuel feedstock species that may be grown on marginal lands including those with saline soils. Our study investigated salt stress responses in 46 switchgrass lines from the lowland and upland ecotypes by assessing physiological phenotypes and proline concentrations. Lowland switchgrass lines demonstrated less severe responses to salt stress than most upland switchgrass lines, but a number of upland lines performed as well as lowland individuals. Photosynthetic rate (Pn), the most important physiological trait measured, was reduced by salt treatment in all lines. Tolerant lines showed ~50% reduction in Pn under salt stress, and sensitive lines exhibited ~90% reduction in Pn after salt stress. Proline analysis showed the largest amount of variation under salt stress with some lines exhibiting minor increases in proline, but some salt-sensitive lines demonstrated more than 5000-fold increase in proline concentration in response to salt treatment. Clustering of salt-stress phenotypic responses revealed five groups of switchgrass. Lowland lines were present in two of the phenotypic clusters, but upland lines were found in all five of the phenotypic clusters. These results suggest that there are multiple modes of salt response in switchgrass including two distinct modes of salt tolerance.

KEYWORD INDEX

switchgrass, salt stress, physiology, proline, photosynthetic rate

1. INTRODUCTION

Switchgrass (*Panicum virgatum*) is a North American C4 perennial grass that ranges from southern Canada to northern Mexico and was one of the dominant species of the North American tall-grass prairie (Casler *et al.*, 2011). Switchgrass has traditionally been grown as a forage crop but has also been used for erosion control (Casler, 2012; Lemus *et al.*, 2002). More recently, due to its high biomass production and capacity to grow in a wide variety of environments including marginal lands, switchgrass has been identified for development as a cellulosic biofuel feedstock by the United States Department of Energy Biofuel Feedstock Development Program (Casler *et al.*, 2007; Sanderson, 2007). Ongoing research has focused on improving switchgrass yield and converting switchgrass biomass into biofuel (Casler, 2012; Lemus *et al.*, 2002; Schmer *et al.*, 2008).

Lowland and upland ecotypes of switchgrass are recognized (Porter Jr., 1966). These ecotypes differ in their growth phenotypes and the regions in which they are typically found. Lowland switchgrass are taller and thicker-stemmed and also have later flowering times compared to upland switchgrass (Casler, 2012; Van Esbroeck *et al.*, 1997). The lowland ecotype of switchgrass typically grows in the southern portion of the species' range while the upland ecotype is typically found in more northern latitudes. However, individuals of both ecotypes can be found in close proximity in the middle latitudes of the species' range (Casler *et al.*, 2007; Casler *et al.*, 2004). Previous studies have shown that lowland switchgrass lines are often more drought tolerant than upland lines (Barney *et al.*, 2009; Liu *et al.*, 2015; Stroup *et al.*, 2003). However, while

upland genotypes readily overwinter in their northern range, most lowland plants show poor survivability in cold northern environments (Casler & Vogel, 2014; Palmer *et al.*, 2014).

It is expected that switchgrass that will be used for biofuel production will often be grown on marginal lands such as those that are affected by high salinity. Naturally saline land comprises 3% of arable land throughout the world (FAO, 2000), but it is estimated that more than 10% of all irrigated farmland worldwide may have harmful levels of salt due to irrigation (FAO, 2002). In the United States, 5.9 million hectares of land are estimated to be salt-affected (Abrol *et al.*, 1988). Areas of the Great Plains of the United States are naturally affected by salinity, and there is concern that changing weather patterns will increase this problem (Brown *et al.*, 1982; Lobell *et al.*, 2010). Salinity can greatly reduce plant productivity (Apse *et al.*, 1999; Bandeoglu *et al.*, 2004; Boyer, 1982; Hosseini *et al.*, 2015; Richter *et al.*, 2015), and therefore, the characterization of the breadth and nature of responses to salt stress that can be found within switchgrass will be useful for breeding efforts.

The physiological response to salt stress is complex, and therefore, there are many opportunities for phenotypic variation. The response to salt stress by plants occurs in two stages (Munns *et al.*, 1995). There is an initial osmotic response that is the result of the change in ionic concentration in the soil media. The osmotic response causes a decrease in growth rate due to lower turgor pressure in expanding cells. Leaf water potential is also reduced which causes stomatal pores to close thereby reducing photosynthetic rate (P_n), transpiration rate (T_r), stomatal conductance (g_s), intercellular

CO₂ concentration (Ci), leaf relative water content (RWC) and water use efficiency (WUE). The second stage of salt stress is an ionic response that occurs as Na⁺ ions begin to accumulate in leaf tissues. Na⁺ is partitioned into the cellular tonoplasts and is actively removed from organelles (Apse *et al.*, 1999; Muller *et al.*, 2014). Osmotically compatible metabolites are also synthesized to help compensate for the reduced water potential in the cytoplasm and organelles (Mattioni *et al.*, 1997; Richter *et al.*, 2015). However, as Na⁺ concentrations continue to increase, membrane integrity will become compromised, and this can be detected by measuring electrolyte leakage (EL). A result of both the osmotically induced reduction in photosynthetic rate and Na⁺-mediated membrane leakage is an increase in reactive oxygen species (ROS), and a number of biosynthetic and enzymatic pathways are activated to handle ROS (Bandeoglu *et al.*, 2004; Cheng *et al.*, 2015; Jain *et al.*, 2015; Shu *et al.*, 2015; Wu *et al.*, 2015; Wutipraditkul *et al.*, 2015).

Proline accumulation in response to salt stress is also observed during other abiotic stress conditions such as drought (Gregorova *et al.*, 2015; Hasegawa *et al.*, 2000; Maritim *et al.*, 2015; Muscolo *et al.*, 2015; Yancey, 2005; Yoshida *et al.*, 1997), and proline is believed to have a two-fold role during salt and drought stresses. First, evidence shows that proline can act as an osmoticum to compensate for low turgor pressure within the cell (Delauney *et al.*, 1993; Voetberg & Sharp, 1991), although there is some question about this role of proline (Borgo *et al.*, 2015; Delauney & Verma, 1993). Second, proline reduces ROS under salt and drought conditions by an unknown mechanism (Hong *et al.*, 2000; Wutipraditkul *et al.*, 2015). Recent work has indicated

that the catabolism of proline is also required for lowering ROS during drought stress (Sharma *et al.*, 2011; Szekely *et al.*, 2008). This understanding of the dual requirement for increased proline levels and proline catabolism may explain reports where proline levels are not positively correlated with salt tolerance (Aziz *et al.*, 1998; Montesinos-Pereira *et al.*, 2014).

Previous studies of salt stress responses in switchgrass measured changes in yield, germination rate, plant height, photosynthetic rates, biomass accumulation and compensating metabolite production (Anderson *et al.*, 2015; Dkhili & Anderson, 1990; Greub *et al.*, 1985; Kim *et al.*, 2012; Liu *et al.*, 2014a; Zhuo *et al.*, 2015). These reports found that switchgrass exhibits reduced growth under salt stress and that lowland lines are more tolerant than upland switchgrass, but they only examined one or a few lines each, at most. Recently, (Liu *et al.*, 2014b) surveyed salt stress responses in seedlings of 33 switchgrass lines by assessing P_n , T_r , g_s , EL, chlorophyll content and photochemical efficiency. The analysis of that large collection of switchgrass lines allowed Liu et al (2014) to show that there is considerable variation in salt response within switchgrass. Clustering analysis based on measured physiological responses to salt stress found two main groups within the 33 switchgrass lines. One group contained only upland lines characterized as sensitive to salt stress. The other group contained upland and lowland lines and contained the most salt tolerant lines.

In this study, we examined switchgrass lines after 30 days of salt stress and extended the work of Liu et al (Liu *et al.*, 2014b). We examined a total of 46 lowland and upland switchgrass lines using a different suite of physiological analyses, and we

also measured changes in proline in response to salt stress. We identified salt tolerant and salt susceptible switchgrass lines. These results provide an indication of the variability of salt responses within switchgrass, which will be useful to breeders. We also describe cluster analysis of the 46 switchgrass lines using the physiological and proline measurements, and this result suggests that there are multiple mechanisms in switchgrass that lead to either tolerance or sensitivity to salt stress and that these mechanisms may not be delineated by ecotype designations. We propose that these complex responses to salt stress by switchgrass indicate that switchgrass may provide a useful system for studying salt stress responses in plants.

2. MATERIALS AND METHODS

2.1 Plant material, growth and salt treatment

The 46 switchgrass genotypes obtained from the United States Department of Agriculture Germplasm Center that were used in this study are listed in Table 1. Sods of each switchgrass genotype were collected from field plots in the Virginia Tech Horticulture Farm, Virginia, USA and were transplanted into plastic pots (17 cm diam., 20 cm height) filled with 3 kg sand. The plants were grown in a greenhouse with a temperature regime of $30\pm1/20\pm1^{\circ}\text{C}$ (day/night), a 12-h photoperiod, 75% relative humidity, and with photosynthetically active radiation of approximately $500\text{ }\mu\text{mol m}^{-2}\text{s}^{-1}$ (natural daylight supplemented with fluorescent lamps). Plants were irrigated daily, and fertilizer (Bulldog brand, 28-8-18, 1% ammonia N, 4.8% nitrate N, 22.2% urea N and

micronutrients; SQM North America, Atlanta, GA) was applied at 0.49 kg m^{-2} every week.

Plants at the E5 developmental stage (Hardin *et al.*, 2013) were irrigated daily either with water (control with electrical conductivity (EC) = 1 dS m^{-1}) or with 250 mM NaCl solution (EC $\approx 21 \text{ dS m}^{-1}$) to induce salinity stress for 30 d. The NaCl concentration was gradually increased to 250 mM by adding 60 mM NaCl every 12 h to avoid salt shock. Previous work with perennial grasses, including switchgrass, has shown that 250 mM NaCl elicited notable salt stress responses, and this level of salt allowed the differentiation of tolerant and sensitive genotypes (Hu *et al.*, 2012; Liu *et al.*, 2014b). The solution with NaCl was slowly watered into the sand, and care was taken to avoid salt solution on leaves, minimizing potential salt-induced leaf burning. To keep the salt concentration constant in the pots during the stress period, salt solution was provided daily by drip irrigation until drainage occurred from the bottom of the pots. A split plot design was used with control/salt treatments as the main plots and switchgrass lines as the subplots. Each line had six replicates for each salt level (0 and 250 mM NaCl). Physiological measurements and proline sampling were performed on treatment day 30 as while some salt-tolerant lines were still relatively healthy, many salt-sensitive lines were exhibiting signs of stress with some beginning to senesce (data not shown).

2.2 Physiological measurements

On treatment day 30, P_n , T_r , g_s , C_i and WUE measurements were determined for control and salt treated plants. Additionally, leaf samples were collected for leaf EL, RWC and proline analyses at the same time. A LiCor Li-6400XT photosynthesis system

(LICOR, Lincoln, NE, USA) was used to measure P_n , T_r , g_s and C_i on the youngest fully expanded leaf on the main tiller of each plant under controlled atmosphere (385 $\mu\text{mol mol}^{-1}$ CO_2 , 500 $\mu\text{mol s}^{-1}$ flow rate, 26 °C) while using a Licor 6400 LED external light source (2000 $\mu\text{mol m}^{-2}\text{s}^{-1}$). Three readings were collected for each sample and the average was used for statistical analysis. WUE measurements were calculated from P_n and T_r ($\text{WUE} = P_n / T_r$). The method from (Marcum *et al.*, 1998) was used to measure leaf EL. The top second or third mature leaf blades were excised, cut into 2 cm segments, rinsed repeatedly with deionized water. Leaf segments (0.2 g) were submerged in a test tube containing 20 mL of deionized water, and the samples were shaken for 24 h. A conductivity meter (SR60IC, VWR, Radnor, PA) was used to assay the solution conductivity (C_1). The tissues were autoclaved (120 °C) for 30 min in the original 20 ml of water, and conductivity after heat treatment (C_2) was measured after the solution cooled. EL was calculated using the formula: $\text{EL}(\%) = (C_1 / C_2) \times 100$. For RWC, the top second or third fully mature leaf blades were sampled and massed to find fresh weight (FW). Turgid weight (TW) was determined after sampled leaves soaked in distilled water for 24 h at 20°C. Leaves were then dried 85°C for 3 d to determine dry weight (DW). RWC was calculated as $\text{RWC} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$ (Barrs & Weatherley, 1962).

Leaf blade samples for metabolite analysis were harvested and immediately frozen with liquid nitrogen. Replicate samples were taken from three different plants from each genotype. The tissue samples were stored at -80 °C until they were shipped

on dry ice to Michigan State University where they were again stored at -80 °C until proline extractions were performed.

2.3 Proline extraction and quantification

Plant tissues frozen in liquid nitrogen were lyophilized overnight and transferred to 2 ml screw cap tubes containing three 3.2 mm stainless steel beads. The tissue was ground, and approximately 50 mg was automatically transferred to a new tube using the iWall tissue-handling robot at the GLBRC Cell Wall facility at Michigan State University. Chemical extraction was performed using 10% methanol and 1% acetic acid solvent containing 10 μ M [2 H $_3$]proline (CDNisotopes, Pointe-Claire, Quebec, Canada) as the internal standard. Approximately 50 Mg of ground plant tissue was extracted by incubation with 400 μ l of extraction solvent at 70°C for 30 min. The extract was centrifuged for 15 min with 13,000 rpm and 200 μ l of supernatant was transferred to a new 1.5 ml microcentrifuge tube. Throughout the entire extraction procedure, all materials were barcoded to ensure reliable sample tracking.

Proline identification and quantification was based on published methods (Gu *et al.*, 2007). Extracts (10 μ l) were injected to a Symmetry C18 column (2.1 x 100 mm, 3.5 μ M particle size, Waters, Milford, MA, USA) attached to a Shimadzu (Columbia, MD, USA) LC-20AD HPLC system for LC reverse-phase analysis. Column temperature was maintained at 30°C. A steep gradient was executed between solvent A and B (A: 1 mM perfluoroheptanoic acid in MilliQ water, B: acetonitrile) with an analysis time of 6 min/sample with 0.3 ml/min flow rate. The gradient profile was 2% B for initial step; linear gradient to 20% B to 0.1 min; 80% B to 2.5 min; 80% B maintained to 4 min; 20% B

to 4.1 min; 2% B to 6min. Mass spectra were acquired using electrospray ionization in positive ion mode and MRM. Quattro micro mass spectrometer (Waters) was coupled to LC to identify and detect analytic signals under the following conditions: electrospray negative ionization mode; 3.17 kV capillary voltage; 110°C source temperature; 350°C desolvation temperature; 20 L/h nebulizer nitrogen flow rate; 400 L/h desolvation nitrogen gas flow rate. Transitions from precursor molecules to characteristic product ions were monitored for proline (m/z 116 > 70) and [$^2\text{H}_3$]proline (m/z 119 > 73). Collision energies and source cone potentials were optimized for each transition using Waters QuanOptimize software.

Peak areas were integrated using the combination of m/z and retention time information characteristic for proline and [$^2\text{H}_3$]proline using QuanLynx v4.1 software (Waters). The amount of observed proline was computed based on the ratio of peak area of the proline to that of [$^2\text{H}_3$]proline, whose concentration was known. The amount was further normalized by the tissue weight used during metabolite extraction.

2.4 Experimental and statistical analyses

For the phenotypic traits investigated in this study, measurements were collected for biological replicates of each switchgrass line, and median absolute deviation (MAD) and median values were calculated. Due to the high variation among samples due to biological and technical factors, outliers were filtered out using median $\pm$ 2 MAD as upper/lower cutoffs. The values within the range of median $\pm$ 2 MAD were used to compute average and standard error for each line. The average trait values were $\log_2$ transformed and then used for multivariate analyses including principal

component analysis (PCA) and hierarchical analysis (HCA). Multivariate analyses were performed and the results were visualized using R (v3.1.1). The salt tolerance index was calculated using the formula: $STI (\%) = \text{response under salt treatment} / \text{response under control condition} \times 100$ (Ali *et al.*, 2007).

3. RESULTS

3.1 High variation in switchgrass responses to salt stress

For most traits (P_n , T_r , g_s , WUE and proline), there was wide variation in observed values between lines under control conditions, but the observed variation between lines was minimal for EL, Ci and RWC (Figure 1, Table S1). We observed high variation between lines in the traits examined under salt conditions (Figure 1, Table S1), consistent with results from previous studies that indicated non-uniform responses to salt by different switchgrass lines (Anderson *et al.*, 2015; Dkhili & Anderson, 1990; Greub *et al.*, 1985; Kim *et al.*, 2012; Liu *et al.*, 2014a; Liu *et al.*, 2014b; Zhuo *et al.*, 2015). Average proline concentration showed the greatest relative variation, and it ranged from 41.5 to 828.7 ng/mg dry weight under control conditions and from 200.5 to 9897.8 ng/mg dry weight under salt treatment, revealing approximately 20- and 50-fold differences between lines in control and salt responses, respectively (Figure 1A, Table S1). Average P_n also varied dramatically between lines. P_n under control conditions ranged from 12.4 to 29.5 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, while it was 0.9 to 9.8 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ under salt stress. Despite the variation, the level of P_n was always higher under control than under salt stress. We used multivariate analysis of all trait measurements to determine

whether informative patterns could be observed between and within salt-treated and control plants. Principal component analysis (PCA) was consistent with the variation that was observed between lines under control and salt conditions. Control samples were more tightly clustered than salt samples (Figure 2A), and lowland and upland lines were not distinctly separated in the control samples. However, lowland lines were mostly separated from upland lines in the salt treated samples. The loading plot for the PCA shows that the cluster of salt-treated samples was positively associated with high EL and proline accumulation but that P_n , T_r , g_s , C_i , WUE and RWC were positively associated with the control samples. WUE had the largest effect on the second principal component, but the significance of the second principal component is unclear as there was no apparent pattern in the distribution of treatments or switchgrass lines along that axis of the PCA plot (Figures 2A and 2B; Table 2).

The P_n , T_r , g_s , C_i , RWC, WUE, EL and proline data were also examined by HCA in order to better visualize any associations between lines, treatments and measured phenotypes. The results clearly showed two clusters: group I with all control plants and group II salt-treated plants (Figure 3, Table S2). The tendency of higher EL and proline accumulation and decreased physiological measurements for RWC, P_n , T_r , g_s , C_i and WUE in salt-treated plants are evident in the HCA plot. Again, the predominant feature in the HCA was the separation of control and salt samples. Notably, lowland lines were dispersed throughout both the control and salt samples although there was more clustering of lowland lines within the salt treated samples.

3.2 Salt tolerance indices distinguish tolerant and susceptible switchgrass lines

While the analysis of the physiological traits and proline levels under control and salt conditions revealed the degree of variation that can be found between switchgrass lines, those analyses were not sufficient to assess which switchgrass lines were the most tolerant or the most susceptible to salt stress. To gain better insight into salt tolerance and susceptibility, salt tolerance indices (STI) were computed for each trait across all lines. The STI results shown in Figure 1 are sorted by increasing STI values. Because STI is calculated as the ratio of a phenotypic measurement under salt stress to that of control, an STI value higher than 100% indicates an increase of the measurement under salt condition compared to control, and an STI value lower than 100% means a decreased value under salt relative to the control condition. As expected, a high diversity of STIs was detected across the measured physiological traits and proline (Figure 1). In most cases, the lowland lines performed better than most upland lines with STI values closer to 100%. Notably, in the key measure of P_n , ten of the best twelve STI values were from lowland lines. Conversely, there were upland lines that performed as well as lowlands in response to salt stress, as determined by STI values for several physiological measurements (Figure 1).

We performed multivariate analysis using the STI values for all switchgrass lines using all physiological traits as well as proline levels. The STI-based PCA scores plot clustered lowland lines closely to each other. The upland lines were separated from the lowland lines but also widely dispersed from each other. Additionally, the first principal component separates samples by ecotype and by tolerance to salt stress (Figure 4A). The STI-based PCA loading plot (Figure 4B) was similar to the PCA loading plot that was

calculated for the salt and control samples (Figure 2B) with the one exception. For the STI-based PCA loading plot, C_i was not well correlated with P_n , T_r , g_s and RWC, but WUE did correlate with P_n , T_r , g_s and RWC (Figures 2B and 4B).

3.3 Hierarchical clustering analysis of switchgrass lines using salt tolerance index

HCA was used to cluster switchgrass lines using the STI values. Because the range of STI values was highly variable for proline levels compared to that of the other physiological traits, all STI values were $\log_2$ transformed before HCA was performed. Consistent with the results above, STIs for EL and proline grouped together, whereas STIs for P_n , T_r , g_s , WUE, RWC and C_i were more similar to each other (Figure 5). The STI-based HCA also showed the 46 switchgrass lines into five clusters (Figure 5, groups I to V). For example, group I showed mild degree of changes in the phenotypic traits under salt stress as indicated by predominantly average STI Z-scores. Group II was characterized by good salt tolerance as indicated by relatively high P_n , T_r , g_s , WUE, RWC and C_i STI Z-scores and relatively low EL STI Z-scores. Groups III and IV may be described as susceptible to salt stress as shown by poor P_n , T_r , g_s , WUE, RWC and C_i STI Z-scores. However, a major difference between groups III and IV was the STI Z-scores for EL and proline. Group III have higher EL and lower proline STI Z-scores, whereas switchgrass lines in group IV showed lower EL and higher proline STI Z-scores. Like group II, switchgrass lines in group V were also salt tolerant as demonstrated by marginal effects in P_n , T_r , g_s , WUE, RWC and C_i STI Z-scores. However, groups II and V did differ in their STI Z-scores for EL and proline. Group II had low EL and high proline STI Z-scores, but for group V the opposite pattern was found. These results indicate that the switchgrass

lines in groups II and V are the most tolerant of salt stress and that the lines in groups III and IV are the most susceptible to salt stress (Figure 5).

All ten lowland ecotypes belonged to high tolerance groups (II and V), including BN-13645-64, BN-12323-69 and T-2086 in group II and AM-314/MS-15S, BN-11357-63, TEM-SEC, Alamo, TEM-LoDorm, TEM-SLC and Kanlow in group V (Figure 5, Table S3). Unlike the lowland lines, the 36 upland lines were distributed across all five groups (Figure 5, Table S3). The two clusters of switchgrass lines with the poorest physiological responses to salt stress (groups III and IV) contained only upland lines. The group I cluster also contained only upland lines but exhibited moderate physiological responses to salt stress. However, upland lines that are relatively tolerant of salt stress were found in group II (T4613, Caddo, Pathfinder, T-2101) and group V (BN-18758-67, 70SG0023, 70SG0024, 70SG0021).

4. DISCUSSION

This study assessed eight phenotypes across 46 switchgrass cultivars and compared the measured traits between control and salt conditions (Tables 1 and 2). We found a wide variation between switchgrass lines under control conditions for most of the traits examined (Figure 1). This phenotypic diversity is consistent with previous reports on phenotypic variation and adaption to different environmental conditions in switchgrass (Casler, 2012; Casler *et al.*, 2007; Cortese *et al.*, 2010; Vogel *et al.*, 2011). We also observed strong variation in switchgrass lines grown under salt stress, demonstrating differential salt stress responses in this collection of switchgrass cultivars

(Figure 1). Compared to control conditions, salt stress resulted in decreases in P_n , T_r , g_s , C_i and RWC but increases in EL and proline measurements (Figures 1). These findings are consistent with expectations based on previous research showing decreased capacity of photosynthesis and plant damage under salt stress salt stress in switchgrass (Anderson *et al.*, 2015; Dkhili & Anderson, 1990; Greub *et al.*, 1985; Kim *et al.*, 2012; Liu *et al.*, 2014a; Liu *et al.*, 2014b; Zhuo *et al.*, 2015). Most switchgrass lines exhibited decreased WUE in response to salt, but in a few lines, WUE was not affected by salt treatment (Figures 1).

Given that we did not observe uniform responses in P_n , T_r , g_s , C_i , RWC, WUE, EL and proline measurements across the 46 tested switchgrass lines under control and salt conditions, we used both PCA and HCA to attempt to identify correlative patterns that might provide insight into the mechanisms that are used by switchgrass in response to salt stress. In the PCA performed with the combined control and salt trait data, the control samples were very tightly clustered but distinctly separated from the salt samples, but within the salt samples in that PCA the lowland lines were clustered and separated from the upland lines (Figure 2A). Similarly, the STI PCA also clearly separated all lowland lines from the upland lines (Figure 4A). Both of these results suggest that as a group the lowland lines in this study are physiologically distinct from the uplands in their response to salt stress. Furthermore, the PCA plots also suggest the relative tolerance of the upland lines to salt stress. The upland lines that are closest to the lowland cluster in the PCA did tend to be tolerant to salt treatment in the individual trait measurements, and the upland lines that are furthest from the lowland cluster were the

most sensitive to salt treatment in the individual trait measurements (Figures 1, 2A and 4A).

Multiple studies have reported on the relative tolerance of various switchgrass lines to salt stress, and it has been shown that a wide variation in responses to salt can be demonstrated within switchgrass (Anderson *et al.*, 2015; Dkhili & Anderson, 1990; Greub *et al.*, 1985; Kim *et al.*, 2012; Liu *et al.*, 2014a; Zhuo *et al.*, 2015). Most of these studies only examined the effect of salt on a few traits such as biomass, seedling emergence rate, seed germination, plant height, proline, malondialdehyde or sugar content. When comparisons between lowland and upland switchgrass have been made, lowland lines have been found to be more tolerant of salt stress than upland lines. Recently, Liu *et al.* (Liu *et al.*, 2014b) measured changes in P_n , T_r , g_s , EL, chlorophyll content and leaf photochemical efficiency in response to salt treatment, and that study found wide variation in the tolerance of 33 switchgrass lines to salt stress. Additionally, Liu *et al.* (Liu *et al.*, 2014b) identified tolerant lines from both lowland and upland ecotypes. Similarly, in this study, we found all ten lowland lines to have relatively high tolerance under salt stress (Figure 1). However, both tolerant and susceptible cultivars were found from upland ecotypes. Because only a limited number of lowland lines were investigated in this study, it is possible that lowland populations exist that are also notably susceptible to salt stress. A more extensive survey of switchgrass populations would be required to more completely understand salt stress responses between and within ecotypes.

Clustering was also performed using HCA with the STI Z-score data to further explore the relationships between the switchgrass lines. The STI HCA resulted in five groups of cultivars with differential responses to salt stress (Figure 5). Two of these groups (II and V) contain the most salt tolerant lines but are predominantly distinguished by differing responses of EL and proline accumulation, but both of these groups contained both upland and lowland switchgrass lines. This result differed from the PCA where the lowland switchgrass lines were clearly distinguished from the upland lines. The existence of these differing patterns of response in EL and proline accumulation, while other physiological traits, including P_n , exhibit relatively minor effects of salt stress, suggests that there exist multiple modes for responding effectively to salt stress in switchgrass.

The STI Z-score HCA results differ notably with the clustering analysis by (Liu *et al.*, 2014b) when using a similar collection of switchgrass lines. While we also found five response groups when analyzing the STI Z-score data by HCA. Liu *et al.* (2014) only found two response groups when clustering their STI data by HCA, and lowland lines were only present in one group. In the analysis of the STI results from the current work, lowland lines were distributed between two different response groups. These differences are likely due to the inclusion of Ci, RWC, WUE and proline analyses in this study, and indicate that other research involving screening for salt responses might also benefit from surveying a larger set of phenotypes.

It is known that proline biosynthesis can be stimulated in response to salt stress (Hasegawa *et al.*, 2000; Yancey, 2005; Yoshida *et al.*, 1997), and we also observed

increased proline concentrations in salt stressed switchgrass (Figure 1A). However, the most salt tolerant lines demonstrated a much lower relative increase in proline levels than did the most salt sensitive lines. This result puts switchgrass in the class of species where proline levels are not positively correlated with salt tolerance (Aziz *et al.*, 1998; Montesinos-Pereira *et al.*, 2014). Given the observation that proline catabolism is required for stress-induced ROS compensation (Sharma *et al.*, 2011; Szekely *et al.*, 2008), we would hypothesize that proline catabolism may also be important for salt tolerance in switchgrass and that those switchgrass lines with the highest accumulation of proline may suffer from the inability to productively catabolize proline. At the same time, it may also be possible that proline catabolism is involved in salt tolerance in those switchgrass lines that demonstrate salt tolerance but that also have lower levels of salt-induced proline. Alternatively, more metabolic energy may be required for biosynthesis of nitrogen-rich compounds (such as proline) relative to carbon-rich compounds (carbohydrates), and salt tolerant switchgrass lines may use less metabolic energy for osmotic adjustment in response to salt stress by maintaining a relatively lower level of proline and a relatively higher level of carbohydrates under salt stress.

Our findings of diverse salt response in switchgrass are helpful in multiple ways. First, we described the variability of responses to salt stress in switchgrass, and this information will be of interest to breeders. Second, we found that while lowland switchgrass were generally very tolerant of salt stress, there were upland switchgrass lines that were also very salt tolerant, and this should indicate to breeders who work with upland lines that abiotic stress tolerant material can be found within upland

germplasm. Third, the recognition of multiple response modes to salt stress in switchgrass suggests that it may be possible to cross salt-tolerant switchgrass lines to further improve salt tolerance in switchgrass. Finally, the observation that high proline content does not necessarily correspond to salt tolerance in switchgrass leads us to predict that proline catabolism may be deficient in at least some salt sensitive switchgrass lines, and points to the need to explore whether or not salt-tolerant lines with low salt-induced proline levels may be catabolizing proline as part of their salt-tolerance response.

AUTHOR CONTRIBUTIONS

KLC, BZ and XZ conceived of the project and designed the experiments. YL and XZ performed the experiments. JK and YL analyzed the data. JK, YL and KC wrote the manuscript with input from BZ and XZ.

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Figure legends

Figure 1. Phenotypic traits assessed at day 30 in this study. Salt tolerance indices (STIs) are presented for traits, (A) proline (B) EL, (C) C_i , (D) WUE, (E) P_n , (F) RWC, (G) T_r and (H) g_s with averages and standard errors under control and salt treatment for individual cultivars. Cultivars are presented following the order of STIs for each trait. Lowland ecotypes are marked with arrows. All measurements are shown in Table S1.

Figure 2. Principal component analysis (PCA) of physiological traits and proline distinguishes salt-treated plants from control plants. PCA was performed with $\log_2$ -transformed average for phenotypic traits, and the results are plotted with the first and second principal components (PC1 and PC2). **A.** Scores plots: control plants are labeled with black fonts, whereas salt-treated plants with red fonts. Cultivar names are labeled with alphabet characters and listed in Table 1. Seven lowland ecotypes (labeled as A to G) are inside the shaded areas for control and salt treatment. **B.** Loadings plots: loadings that contribute the separation between salt-treated and control plants are marked with circles. Each phenotypic traits are labeled with alphabet characters and listed in Table 2.

Figure 3. Alteration of physiological traits and proline accumulation under salt stress. Heat map was generated with $\log_2$ -transformed average for seven physiological traits and proline and presented as Z-scores. The y-axis represents switchgrass cultivar names that are listed in Table S2 for clarity in this figure. Lowland ecotypes are marked with black circles (open: control plants; closed: salt-treated plants).

Figure 4. Principal component analysis (PCA) of salt tolerance indices (STI) from forty-six switchgrass cultivars. PCA was performed with STIs from eight phenotypic measurements. The first two principal components (PC1 and PC2) are plotted. **A.** Scores plots: the identical labels are used as in Figure A. See Table 1 for the list of cultivar names. Seven lowland ecotypes (labeled as A to G) are inside the shaded area. **B.** Loadings plots for the PCA. Trait labels are described in Table 2.

Figure 5. Heat map derived from hierarchical clustering analysis of switchgrass salt tolerant indices (STIs). The heat map was generated using Z-score normalized $\log_2$ -transformed STIs. Cultivar names are listed in Table S3. Lowland ecotypes are marked with black circles.

Table 1. The list of forty-six switchgrass lines investigated in this study.

Accession	Plant ID	Ecotypes	Label²
PI 421999	AM-314/MS-155	Lowland	A
PI 422006	Alamo	Lowland	B
PI 315728	BN-13645-64	Lowland	C
PI 421521	Kanlow	Lowland	D
PI 636468	TEM-LoDorm	Lowland	E
PI 607838	TEM-SEC	Lowland	F
PI 607837	TEM-SLC	Lowland	G
PI 642193	70SG001	Upland	H
PI 642207	70SG0016	Upland	I
PI 642208	70SG0017	Upland	J
PI 642209	70SG0018	Upland	K
PI 642210	70SG0019	Upland	L
PI 642194	70SG002	Upland	M
PI 642211	70SG0020	Upland	N
PI 642212	70SG0021	Upland	O
PI 642213	70SG0022	Upland	P
PI 642214	70SG0023	Upland	Q
PI 642215	70SG0024	Upland	R
PI 642195	70SG003	Upland	S
PI 642196	70SG004	Upland	T
PI 642197	70SG005	Upland	U
Grif 16407	Blackwell-1	Upland	V
Grif 16409	Blackwell-2	Upland	W
PI 421520	Blackwell-3	Upland	X
PI 315724	BN-10860-61	Upland	Y
PI 315727	BN-11357-63	Lowland	Z
PI 414070	BN-12323-69	Lowland	a
PI 414068	BN-18758-67	Upland	b
PI 414067	BN-8624-67	Upland	c
PI 476297	Caddo	Upland	d
PI 537588	Dacotah	Upland	e
PI 414066	Grenville-2	Upland	f
Grif 16408	Grif Nebraska 28 ¹	Upland	g
PI 477003	Nebraska 28	Upland	h
PI 642192	Pathfinder	Upland	i
PI 591824	Shawnee	Upland	j
PI 642191	Summer	Upland	k
PI 598136	Sunburst	Upland	l
PI 476290	T-2086	Lowland	m
PI 476292	T-2100	Upland	n
PI 476293	T-2101	Upland	o
PI 476296	T16971	Upland	p
PI 476295	T4613	Upland	q
PI 476294	T4614	Upland	r

PI 549094	Trailblazer	Upland	s
PI 204907	Turkey	Upland	t

¹Grif Nebraska 28 is labeled as GNebraska 28 throughout figures in this manuscript.

²Labels are identical to the labels used in the score plots in Figures 2 and 4.

Table 2. The list of traits measured in this study. Labels are identical to the labels used in the loading plots in Figures 2 and 4.

Traits and abbreviations	Label
Leaf relative water content (RWC)	A
Electrolyte leakage (EL)	B
Photosynthetic rate (Pn)	C
Stomatal conductance (Gs)	D
Transpiration rate (Tr)	E
Intercellular CO ₂ concentration (Ci)	F
Water use efficiency (WUE)	G
Proline	H

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5