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Permalink

https://escholarship.org/uc/item/5w3948zr

Journal

Journal of agricultural and food chemistry, 63(8)

ISSN

0021-8561

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Publication Date

2015-03-01

DOI

10.1021/jf503948p

Peer reviewed

Effects of Timing and Severity of Salinity Stress on Rice (*Oryza sativa* L.) Yield, Grain Composition, and Starch Functionality

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Ⓢ *Supporting Information*

ABSTRACT: The aim of this work was to examine agronomic, compositional, and functional changes in rice (*Oryza sativa* L. cv. Nipponbare) grains from plants grown under low-to-moderate salinity stress in the greenhouse. Plants were grown in sodium chloride-containing soil (2 or 4 dS/m^2 electrical conductivity), which was imposed 4-weeks after transplant (called Seedling EC2 and EC4) or after the appearance of the anthers (called Anthesis EC2 and EC4). The former simulates field conditions while the latter permits observation of the isolated effect of salt on grain filling processes. Key findings of this study are the following: (i) Plants showed adaptive responses to prolonged salt treatment with no negative effects on grain weight or fertility. Seedling EC2 plants had more panicles and enhanced caryopsis dimensions, while surprisingly, Seedling EC4 plants did not differ from the control group in the agronomic parameters measured. (ii) Grain starch increased in Seedling EC4 (32.6%) and Anthesis EC2 (39%), respectively, suggesting a stimulatory effect of salt on starch accumulation. (iii) The salinity treatment of 2 dS/m^2 was better tolerated at anthesis than the 4 dS/m^2 treatment as the latter led to reduced grain weight (28.8%) and seed fertility (19.4%) and compensatory increases in protein (20.1%) and nitrogen (19.8%) contents. (iv) Although some salinity treatments led to changes in starch content, these did not alter starch fine structure, morphology, or composition. We observed no differences in reducing sugar and amylose content or starch granule size distribution among any of the treatments. The only alterations in starch were limited to small changes in thermal properties and glucan chain distribution, which were only seen in the Anthesis EC4 treatment. This similarity of compositional and functional features was supported by multivariate analysis of all variables measured, which suggested that differences due to treatments were minimal. Overall, this study documents the specific response of rice under defined conditions, and illustrates that the plasticity of plant response to mild stress is complex and highly context-dependent, even under greenhouse conditions in which other potential environmental stress impacts are minimized.

KEYWORDS: *rice (Oryza sativa L.), salinity stress, starch, starch functionality*

■ INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops as it is a staple for over 3 billion people globally.[1] Starch, and to a lesser extent protein, determine grain yields and quality, two important criteria for rice producers.[2] Yield stability is the overriding agronomic consideration in places where rice is critical for food security, while grain sensory attributes are an important driver in other markets.[2,3] Rice yield and sensory quality are both affected by environmental stress, and there is increasing concern on the specific effects of both.[4−8] Soil salinity is one such environmental factor that is increasing in magnitude in many rice growing regions due to a combination of drought, high temperatures, rising sea levels, and poor agricultural practices.[9] Under field conditions, yield reductions of one-third can occur when rice is grown at an electrical conductivity (EC) of 2 dS/m^2[10] and halved at 4 dS/m^2; these are considered low and moderate salinity stresses, respectively.[8,10−12] Some studies show that as little as 5 dS/m^2 is lethal for rice.[13] Besides yield reductions, changes in grain characteristics are also expected, although they have not been properly established.[6,7,11,14] This should be addressed since

there are strong economic incentives for producers to supply grain that meets specific market expectations.[15]

The precise effect of salinity on the rice harvest is determined by a complex interaction of several factors including the severity, timing, and duration of the stress.[16] Rice salt-sensitivity fluctuates during the lifecycle, but the effects are most severe at the reproductive and seedling stages.[8,17,18] Sodium chloride (NaCl) is the most pernicious salt affecting plant growth and is often used to induce saline conditions in controlled experiments.[4] Plants may be able to adapt to mild sustained, or short-term moderate salinity stress by reprogramming multiple physiological and developmental processes.[4] Salts accumulated in older leaves may cause premature senescence;[4,19] however, any loss of photosynthate may be offset by a high rate of new leaf production.[20] This, plus increased panicle initiation, greater inflorescence formation, and an accelerated (but shorter) grain

filling period,[20,21] collectively, might maintain normal yields. Salt concentrations above the tolerable threshold would compromise these adaptive processes and lead to lower productivity.[20] It is especially devastating when imposed at grain filling, since at this terminal phase of the lifecycle, the plant has fewer compensatory mechanisms to mitigate the buildup of salt in sensitive tissues.[9,20] The results are increased floret sterility, increased percentage of partially filled and unfilled grain, and lower yield.[3,8,17,22,23] Because of the multiplicity of factors that determine plant response to salinity the outcome may not always be predictable.

Rice growth environment also strongly dictates grain postharvest attributes since the grain is consumed whole with minimal processing.[3,24,25] Starch is the main grain reserve in rice accumulating up to 50−90% of dry weight with protein contributing 5−12% to the total.[26] Starch molecular composition and structure are the major quality determinants influencing grain visual, textural, nutritional, and taste attributes, but protein can also impact sensory properties.[15,26,27]

The effects of heat and drought on rice quality have been widely reported, but data on salinity are limited.[3] Some studies show that moderate to high concentrations (EC 4−8 dS/m^2) of salt reduce amylose[6,7,14] and modulate rice texture, pasting properties, and gel consistency.[6,14,28] These functional changes may be due to disruptions in the grain starch-to-storage protein ratio, amylose content, amylopectin glucan chain distribution, the lipids associated with amylose, starch granule crystallinity, and particle size distribution;[3,29−31] however, other than amylose content, most of these parameters have not been investigated.

Because of the importance of rice as a global commodity, and the potential threat of increasing soil salinity to rice production, a better understanding of how salinity affects rice quality and yield is needed. To work toward this goal we took a comprehensive view of how timing and severity of salinity stress can change some aspects of rice productivity and grain sensory quality. Plants were grown in a controlled greenhouse setting to eliminate extraneous, confounding factors.[3] Nipponbare was used, as it is the model rice ecotype, which should permit comparison with other published research.[32,33] Salinity (NaCl) was imposed at the seedling stage in one batch of rice, and at the reproductive stage in another as we anticipated differences in plant response with the differential timing of salt application.[3] This would also enable greater comparison and interpretability with the limited published data on salinity effects on grain quality, which usually adopt one approach, e.g., salt applied at the seedling stage[6,7] or during grain filling.[12,14] Two salt concentrations, EC 2 and 4 dS/m^2, were used to induce a mild and moderate salt stress rather than a nonagronomic "salt shock".[23] This was intended to allow us to examine plant adaptive responses that could be obscured by a rapid onset of senescence. Finally, we aimed to connect changes in grain sensory parameters to modifications occurring in the starch at the molecular level. Collectively these data could provide a nuanced overview of how grain parameters respond to salinity stress.

■ MATERIALS AND METHODS

Plant Growth Conditions. Rice (*Oryza sativa* L.) cv. Nipponbare were grown in a greenhouse in Davis, CA, from May to October 2013 in 5″ pots with a mixture of UC potting mix (1 peat moss: 1 coarse sand (v:v)) and perlite 9:1 (v:v) each containing Osmocote 17-7-12 controlled release fertilizer (B&T Grower Supply, Inc., Forest Hill,

LA) flooded with water. Salt stress was imposed by adding either 20 or 40 mM NaCl to achieve EC values of 2 and 4 dS/m^2 either at 4-weeks after transplanting (V5 stages, ref 34), described here as Seedling EC2 and Seedling EC4, or at anthesis (R8 stage, ref 34), described here as Anthesis EC2 and Anthesis EC4. The control plants were grown without the addition of NaCl and were maintained at EC0. Once all filled seeds were hardened, the water was drained, and plants were kept in pots for another 7 days before the panicles were harvested. The EC was maintained at 2 and 4 (±15% variation) dS/m^2 until harvest (see Supporting Information Figure S1).

Plant Productivity and Seed Characteristics. The number of panicles per plant, 100-grain weight, total grain weight per plant, and seed fertility (expressed as number of fully filled seeds per 100 seeds) were calculated. To examine seed morphology in a high throughput manner, approximately 150 grains were placed on a scanner, and an image of 600 dpi was taken and then used as an input for WinSeedle (Regents Instrument Inc., Canada). Grain length, width, width-to-length ratio, perimeter, surface area, and volume were recorded.

Starch and Reducing Sugar Measurements. A half-gram of milled grains was homogenized to a fine powder using a Thomas Wiley Mini-Mill (Thomas Scientific, Swedesboro, NJ). Sugars were extracted from the grain by boiling 100 mg of milled flour samples three times in 80% (v/v) ethanol. Ethanol was removed by drying the soluble fraction in a speedvac, and the residue was reconstituted in 300 µL of water. Starch was analyzed from the ethanol-insoluble residue by digesting to glucose as previously described.[35] Reducing sugars in the reconstituted sample soluble fraction, as well as glucose digested from starch, were analyzed using 3,5-dinitrosalicylic acid.[36]

Starch Purification and Analysis. Starch was extracted as described in ref 37 except that 15 g of seeds was used. The samples were homogenized in a blender, and the homogenate was filtered through 4 layers of Miracloth. *Amylose* was assayed on 10 mg of purified starch using the Megazyme amylose/amylopectin determination kit (Wickson, Ireland).[36] *Amylopectin branch chain-length distribution* was analyzed by high-performance anion-exchange chromatography (HPAEC) as previously described.[38] *Thermal analyses* were done by differential scanning calorimetry (DSC), with the method modified from ref 39. Starch slurries with a water-to-starch ratio of 3:1 (~15 mg) were hermetically sealed in a large volume stainless steel pan, and equilibrated overnight at room temperature prior to analysis. An empty stainless steel pan was used as a reference. The sample and reference pans were scanned from 30 to 120 °C at a heating rate of 10 °C/min. *Particle size analysis* was done as described in ref 40. *Pasting properties* of 8% (w/v) starch slurry was measured by AR1000-N Rheometer (TA Instrument, New Castle, DE). The temperature profile for the rheometer, which simulates the temperature profile of a Rapid Visco-Analyzer (RVA), was as follows: hold at 50 °C for 1 min, heat to 95 °C over 4.5 min, hold at 95 °C for 3 min, cool to 50 °C over 4.5 min, and finally hold at 50 °C for 1 min. The experiments were done in triplicate.

Total Protein and Nitrogen Content and SDS-PAGE Profile of Grain Flour. Protein and nitrogen (N) contents were assayed using the AOAC Kjeldahl method.[41] To analyze grains storage protein, 40 mg of flour was homogenized in 700 µL of sodium dodecyl sulfate−urea solution overnight and then centrifuged at 7000 g for 5 min.[42] Then, 5 µL of supernatant was loaded onto 10% (w/v) precast bis-tris sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (Invitrogen, Carlsbad, CA) and subjected to electrophoresis. Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, CA) was used to stain the separated proteins, which were visualized using an AlphaImager (Alpha Innotech, San Leandro, CA). The density of each band was measured using ImageJ (NIH, Bethesda, MA).[43]

Statistical and Multivariate Analysis. A one-way analysis of variance (ANOVA) was used to determine statistically significant differences in means between treatments. A Tukey−Kramer post hoc test was calculated when statistical differences were observed. A minimum of 3, but typically 5−10, biological replicates were used. Differences were deemed to be statistically significant when $P \leq 0.05$. Partial-least squares discriminant analysis (PLS-DA) was done

Table 1. Productivity of Nipponbare Rices Grown under Different Salinity Conditions[a]

treatment	EC (dS/m^2)	panicle no.	% fertility	100 grain wt (g)	total grain wt (g)
control	0	10.3 ± 0.26 b	87.8 ± 0.84 a	2.24 ± 0.02 a	28.79 ± 1.49 a
seedling	2	**12.5 ± 0.60 a**	86 ± 2.60 a	2.38 ± 0.07 a	31.27 ± 2.38 a
	4	11.7 ± 0.54 a,b	80.4 ± 4.51 a,b	2.09 ± 0.11 a,b	26.81 ± 1.21 a,b
anthesis	2	9.6 ± 0.34 b	80 ± 2.02 a,b	2.11 ± 0.05 a,b	28.14 ± 1.19 a
	4	9.3 ± 0.42 b	**70.8 ± 3.41 b**	**1.91 ± 0.09 b**	**20.51 ± 1.95 b**

[a]Values are means ± SEM. Means within each column with different superscripts and presented in bold are significantly different ($P \leq 0.05$) by Tukey's test ($n = 10$).

following data normalization via logarithmic transformation using Metaboanalyst online tool (TMIC, Canada).[44]

RESULTS AND DISCUSSION

The aim of this work was to determine how salinity affects rice productivity and grain quality. These parameters both determine market value and acceptance of novel rice varieties.[14,27,45] Quality parameters encompass not only the physical appearance of the caryopsis, but also the biochemical composition of the grain, and most importantly the physicochemical properties of the endosperm protein and especially starch, as they are the major determinants of rice eating quality.[27] The study also aimed to investigate plants grown in the greenhouse settings where the EC was controlled (Supporting Information Figure S1) in order to achieve clear understanding of how salt impacts grain parameters.

Effect of Saline Soils on Yields and Grain Characteristics. *Plant Productivity.* Changes in rice agronomic yields were assessed by measuring grain weight, caryopsis fertility, and the number of panicles per plant (Table 1). Small and green unfilled caryopses were visible in all salt treatments except the Seedling EC2 (Figure 1). Decreases in fertility, total grain

Figure 1. Rice seeds from control and salinity-treated plants at harvest. There are 100 random seeds of each treatment shown. Several infertile unfilled seeds, as shown by green hull color, were seen in the Seedling 4 dS/m^2, and in the Anthesis treatments from both salt concentrations. The grains from these treatments were also smaller than those from the control and Seedling 2 dS/m^2 treatment (see Table 1).

weight, and 100-grain weight were only statistically significant in the Anthesis EC4 treatment (Table 1). These results were similar to those in a study where EC 3.2 and 4.6 dS/m^2-treated rice led to reductions in grain weight.[16] Reductions of up to 98% in caryopsis weights[6,7,12] and increases in the fraction of unfilled spikelets[12] under high salt (5−8 dS/m^2) have been

reported. The occurrence of unfilled seeds was suggested to be due to reduced activity of starch synthase and decreased translocation of soluble carbohydrate as a result of loss of Na$^+$/ K$^+$ homeostasis in floral organs.[12] Pollen sterility and changes in floral structure may also have contributed to the floret sterility[21] in the Anthesis EC4 treatment. Conversely, Zeng et al. (2001) showed that total grain weight and grain fertility were enhanced when salinity (EC 1.8, 3.2, and 4.6 dS/m^2) was imposed at the booting stage.[16] However, unlike the present study, salinity stress was episodic, only imposed for 20 days after which soil EC was restored to control levels until harvest, perhaps accounting for the positive effects on productivity.[16]

Panicle numbers for the control and both anthesis treatments were not different, while it was enhanced (12%) in the Seedling EC2 treatment (Table 1). This has not been reported in any previous study, and thus requires further investigation to determine whether a mild stress had a stimulatory effect on floral primordia initiation. Since fertility, total grain weight, and 100-grain weight were not affected by the mild level of salt stress, this suggests an agronomically beneficial adaptive response of the plants. From a practical point of view, it should be possible to design experiments to determine the use of low quality irrigation water, or saline soils up to 2 dS/m^2 for rice irrigation purposes, as this treatment has minimal repercussions for rice growth in the greenhouse setting

Grain Characteristics. Grain physical appearance and dimensions are an important indicator of the marketability of rice as they are readily obvious to purchasers;[45] however, the length and shape of the rice kernels also affect kernel milling, cooking, and sensory properties.[46] Here, we showed that prolonged exposure to NaCl throughout development affected grain dimensions (Table 2). Seedling EC4 plants had lower grain volume compared to the control. This could indicate changes in cell number in the lemma and palea because the size of the outer glume greatly restricts the dimension of dehulled rice endosperms.[47] Seedling EC2 plants had increased grain length and surface area (Figure 1 and Table 2). This may be perceived negatively in some Asian markets.[15] On the contrary, a decrease in grain length of two indica rice cultivars has been reported for plants grown in a higher degree of salinity (EC 5−

Table 2. Caryopsis Characteristics of Rices Grown under Different Salinity Conditions[a]

treatment	EC (dS/m^2)	length (mm)	width (mm)	width-to-length ratio	perimeter (mm)	surface area (mm^2)	volume (mm^3)
control	0	4.99 ± 0.04 b	2.98 ± 0.02 a	0.60 ± 0.003 a	13.25 ± 0.09 b	26.11 ± 0.30 b	23.56 ± 0.38 a
seedling	2	**5.14 ± 0.03 a**	2.99 ± 0.02 a	**0.58 ± 0.002 b**	**13.56 ± 0.08 a**	**27.06 ± 0.30 a**	24.53 ± 0.42 a
	4	5.02 ± 0.02 b	2.93 ± 0.01 a	**0.59 ± 0.004 b**	13.27 ± 0.05 b	25.88 ± 0.13 b	**22.98 ± 0.22 b**
anthesis	2	5.12 ± 0.02 a,b	2.98 ± 0.01 a	**0.58 ± 0.003 b**	**13.52 ± 0.05 a**	26.79 ± 0.11 a,b	24.21 ± 0.13 a
	4	5.07 ± 0.02 a,b	2.96 ± 0.01 a	**0.58 ± 0.003 b**	13.41 ± 0.04 a,b	26.39 ± 0.14 a,b	23.68 ± 0.24 a

[a]Values are means ± SEM. Means within each column with different superscripts and presented in bold are significantly different ($P \leq 0.05$) by Tukey's test ($n = 6$). Approximately 100 seeds were scanned for each technical replicates.

Table 3. Storage Product Composition of Rice Caryopses Grown under Different Salinity Conditions[a]

treatment	EC (dS/m²)	reducing sugar content (mg gFW⁻¹)	starch content (mg gFW⁻¹)	N content (%)	protein content (%)	amylose content (%)
control	0	0.13 ± 0.03 a	291.65 ± 6.18 b	1.21 ± 0.05 b	7.18 ± 0.29 b	14.53 ± 0.67 a
seedling	2	0.14 ± 0.02 a	317.57 ± 4.38 b	1.14 ± 0.02 b	6.80 ± 0.14 b	15.59 ± 0.55 a
	4	0.09 ± 0.02 a	**386.85 ± 10.77 a**	1.20 ± 0.04 b	7.14 ± 0.22 b	13.51 ± 0.84 a
anthesis	2	0.19 ± 0.03 a	**405.39 ± 13.77 a**	1.27 ± 0.00 b	7.56 ± 0.05 b	13.63 ± 0.72 a
	4	0.15 ± 0.03 a	319.67 ± 3.98 b	**1.45 ± 0.03 a**	**8.62 ± 0.18 a**	14.69 ± 0.18 a

[a]Values are means ± SEM. Means within each column with different superscripts and presented in bold are significantly different ($P \leq 0.05$) by Tukey's test ($n = 4$ for starch, sugar, and N content analyses; $n = 3$ for amylose content and particle size analyses).

6 dS/m²),[6] again emphasizing that plant response is genotype and environment sensitive.

Grain Storage Product Composition. *Starch, Sugar, and Amylose Contents and Starch Particle Size Distribution.* The major grain carbohydrates are starch, sugars, and amylose. Starch dictates yield, while amylose content is one of the major properties determining rice sensory quality.[48] Differences in starch granule size also influence starch functionality,[49,50] and grain sugars could increase due to stress-induced starch breakdown and may influence taste.[51] Here we found no difference in reducing sugar content (Table 3). Interestingly, we found that the Seedling EC4 and the Anthesis EC2 treatments increased the percentage of starch in the rice flour (Table 3) as well as in each caryopsis (Supporting Information Table S1). These increases in starch content stand in sharp contrast to rice grown under stronger saline conditions (EC 4–8 dS/m²) which reduced endosperm starch[6,14] even in greenhouse grown rice such as ours.[6] Salt enhances starch accumulation in tomato fruits by elevating ADP-glucose pyrophosphorylase (AGPase) activity, a key enzyme in starch biosynthesis.[52] In rice, expression level of the leaf large and small AGPase subunits also increased in response to salinity.[53] However, whether AGPase transcript level and enzyme activity increase in the endosperm in a similar way to that seen in tomato under salinity is worth determining.

Surprisingly, the percentage of amylose in starches from plants exposed to salinity, including those that had increased starch contents, did not differ from that of the control (Table 3). Reductions in amylose exposed to high salt concentration (EC 4–8 dS/m²) at the reproductive stages have been reported;[6,14] however, in one study, salinity exposure at the seedling stage had to be as high as 6–8 dS/m² for amylose content to decrease.[7] The particle size distribution of starch grains was also not affected by salt stress (Supporting Information Table S2). Because salinity stress exposure was associated with increased starch content and no change in the percentage of amylose or starch granule size distribution, it is tempting to speculate that salinity may have altered some aspects of starch biosynthesis such as starch granule initiation and amylose biosynthesis.

Protein and N Content. Rice grain protein and N contents can be increased by salinity stress.[11] In this study, the protein and N content in the flour from the Anthesis EC4 treatment was significantly higher than that of the flours from other growth conditions (Table 3). SDS-PAGE profiles of these grains confirmed that the glutelin and prolamin contents were greatly enhanced (Figure 2). Similarly, six rice cultivars grown under 2.5 dS/m² salinity were found to have higher protein content with major contributions from glutelin.[11] When the protein content was expressed relative to starch content (data not shown), the starch:protein ratio of the Anthesis EC4 sample was lower compared to the control, thus potentially
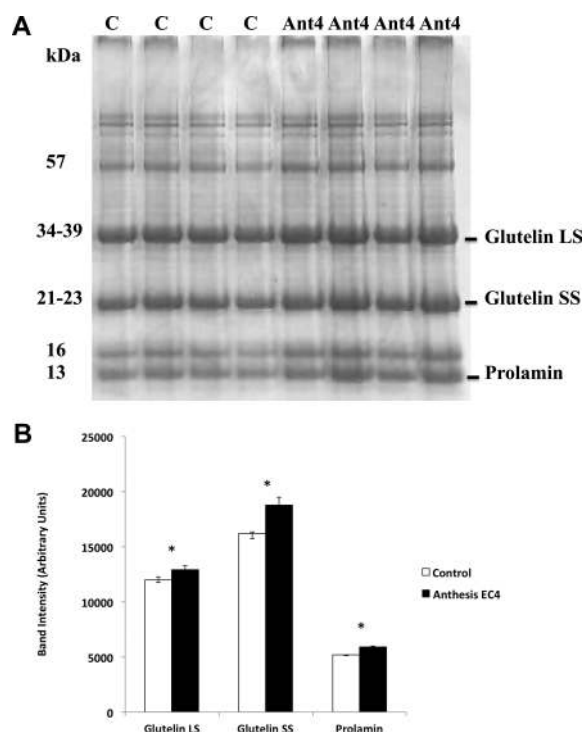

Figure 2. Analysis of grain storage proteins extracted from flour of Control and Anthesis EC4 treatments. (A) SDS-PAGE showing the bands of the glutelin large subunit (LS), glutelin small subunit (SS), and prolamin. (B) Densitometry plot showing the relative content of these rice storage proteins. Values are means ±SEM. Means of each pair of columns with a star are significantly different ($P \leq 0.05$) by Student's *t*-test ($n = 4$).

affecting rice cooking and sensory properties.[54] Removing proteins from rice flours increases the water availability for starch and results in a less viscous product;[54] therefore, the Anthesis EC4 caryopsis, once cooked, might become firmer due to reduced water availability for starch granule swelling and glucan leaching. This high protein-induced hardness in cooked rice may be further amplified because caryopsis amylose content was unchanged (Table 3).[6] However, to determine this, the retrogradation property of the endosperm starch must also be considered.

When expressed on a mg per grain basis, caryopsis protein and starch levels in the Anthesis EC4 treatment were not different from the control (Supporting Information Table S1). The lack of congruency in protein expressed on an equal mass basis versus on a per grain basis suggests differences in reserve allocation and partitioning to the grain due to salinity. Moreover, while grain starch was enhanced in the Seedling EC4 and Anthesis EC2 treatments, the protein accumulation remained stable (Supporting Information Table S1). This
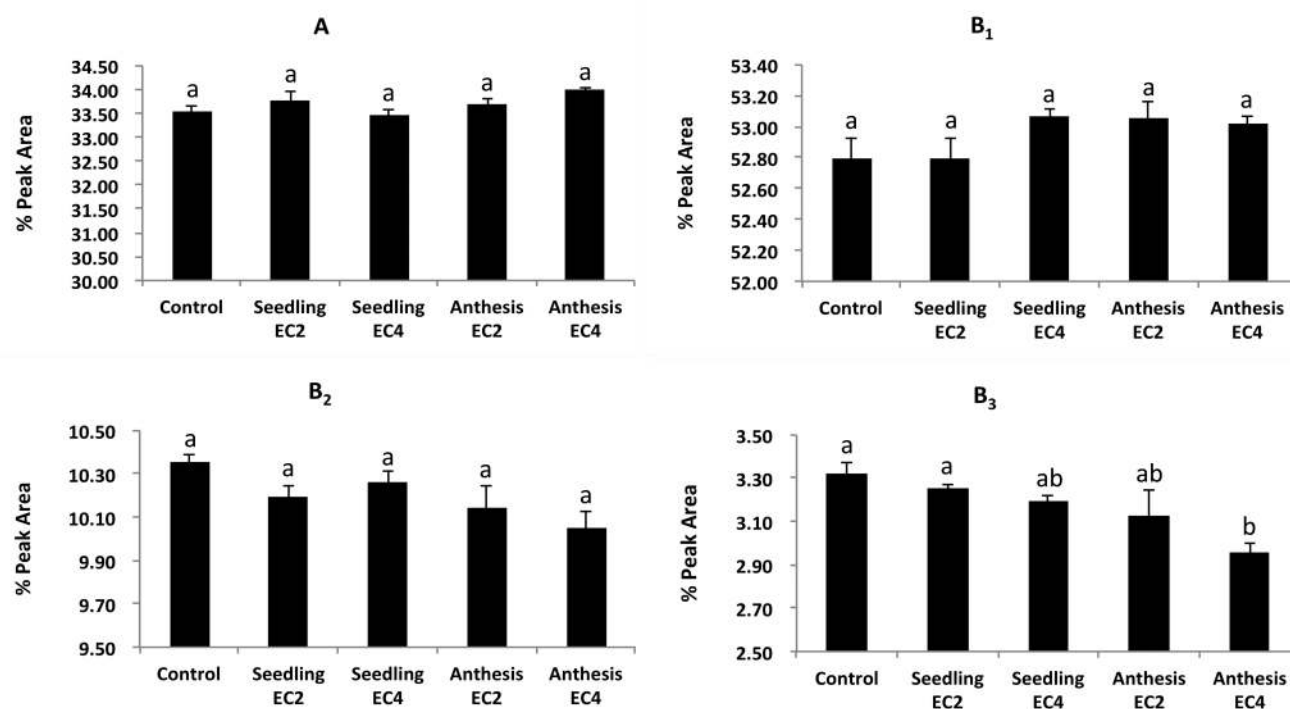
Figure 3. Branch chain length distribution of rice amylopectin of different classes. Starch was digested with isoamylase, and the digested starch was then separated by HPAEC. The distributions of branch chain lengths were categorized into A class (DP 6−12), B_1 class (DP 13−24), B_2 class (DP 25−36), and B_3 class (DP ≥ 37). Values are means ± SEM. Means of each column with different superscripts are significantly different ($P \leq 0.05$) by Tukey's test ($n = 4$).

Table 4. Gelatinization Properties of Rice Starches Analyzed by DSC[a]

treatment	EC (dS/m²)	onset temp (°C)	peak temp (°C)	conclusion temp (°C)	temp range (°C)	enthalpy (J/g)
control	0	61.30 ± 0.36 a	67.41 ± 0.47 a	74.02 ± 0.46 a	12.71 ± 0.63 a	14.62 ± 0.40 a
seedling	2	60.22 ± 0.29 a	**65.69 ± 0.24 b**	**72.04 ± 0.18 b**	11.81 ± 0.38 a	13.28 ± 0.38 a
	4	59.56 ± 0.60 a	65.81 ± 0.50 a,b	**71.95 ± 0.62 b**	12.39 ± 0.17 a	15.66 ± 1.03 a
anthesis	2	60.41 ± 0.22 a	**65.60 ± 0.40 b**	72.63 ± 0.36 a,b	12.23 ± 0.31 a	15.50 ± 0.50 a
	4	60.07 ± 0.39 a	**65.35 ± 0.27 b**	**70.81 ± 0.44 b**	**10.74 ± 0.50 b**	14.24 ± 0.33 a

[a]Values are means ± SEM. Means within each column with different superscripts and presented in bold are significantly different ($P \leq 0.05$) by Tukey's test ($n = 4$).

Table 5. Pasting Properties of Rice Starches[a]

treatment	EC (dS/m²)	pasting temp (°C)	peak viscosity (Pa s)	peak temp (°C)	breakdown (Pa s)	final viscosity (Pa s)	setback (Pa s)
control	0	62.85 ± 0.15 a	0.76 ± 0.08 a	89.83 ± 1.46 a	0.39 ± 0.08 a	0.75 ± 0.06 a	0.38 ± 0.07 a
seedling	2	62.14 ± 0.15 a	0.58 ± 0.03 a	92.02 ± 0.40 a	0.23 ± 0.03 a	**0.51 ± 0.03 b**	**0.15 ± 0.03 b**
	4	62.18 ± 0.21 a	0.74 ± 0.08 a	90.45 ± 0.60 a	0.36 ± 0.08 a	0.65 ± 0.06 a	0.26 ± 0.07 a
anthesis	2	62.53 ± 0.16 a	0.64 ± 0.04 a	91.50 ± 0.29 a	0.25 ± 0.03 a	**0.56 ± 0.04 b**	**0.15 ± 0.03 b**
	4	**61.66 ± 0.28 b**	0.55 ± 0.01 a	91.53 ± 0.39 a	0.22 ± 0.03 a	**0.50 ± 0.02 b**	**0.17 ± 0.02 b**

[a]Values are means ± SEM. Means within each column with different superscripts and presented in bold are significantly different ($P \leq 0.05$) by Tukey's test ($n = 4$).

suggests that, under these two salinity conditions, starch and protein accumulation were differently regulated.

Starch Molecular Structure and Functionality. *Amylopectin Branch Chain-Length Distribution.* Amylopectin fine structure is an important contributor of starch functionality as its branch chain-length distribution is shown to relate to starch crystalline structure[55] and pasting properties.[56] There was no difference in distribution pattern of the branch chains of different degrees of polymerization among all treatment groups (Supporting Information Figure S2), suggesting no notable effect of salinity on the overall amylopectin chain-length profile.

However, when the glucan chain lengths were categorized into discrete classes (A class, DP 6−12; B_1 class, DP 13−24; B_2 class, DP 25−36; and B_3 class, DP ≥ 37),[55] differences were found in the Anthesis EC4 treatment, which had a reduced proportion of the long chains (i.e., DP ≥ 37) (Figure 3).

Starch Gelatinization Properties. DSC enables the heat of gelatinization of a starch−water suspension to be observed as a well-defined endotherm peak.[57] Salt stress reduced both the peak and conclusion temperatures, while the temperature range was smallest in the Anthesis EC4 among others (Table 4). The enthalpy of the system nevertheless did not vary among all

treatments (Table 4). The DSC analysis also reveals the thermal behavior of the amylose–lipid complex melting, which can alter starch functionality.[58] There was no significant difference in the melting profiles of all treatments, except for the Anthesis EC4 treatment, where a significant reduction in onset temperature of melting was found (Supporting Information Table S3). This suggests only a slight difference in strength and amount of lipid complexes with amylose as a result of salinity, and is in agreement with the finding that amylose content was unchanged due to saline conditions in this study.

Starch Pasting Properties. The pasting behavior of a starch/water mixture is measured as a change in viscosity resistance of the mixture to controlled shear as the temperature is increased and decreased.[59] The rheometer-simulated RVA pasting profile showed that Anthesis EC4 starch formed a paste at slightly lower temperatures than the control starch (Table 5 and Figure 4), while the pasting temperatures of other treatments did not
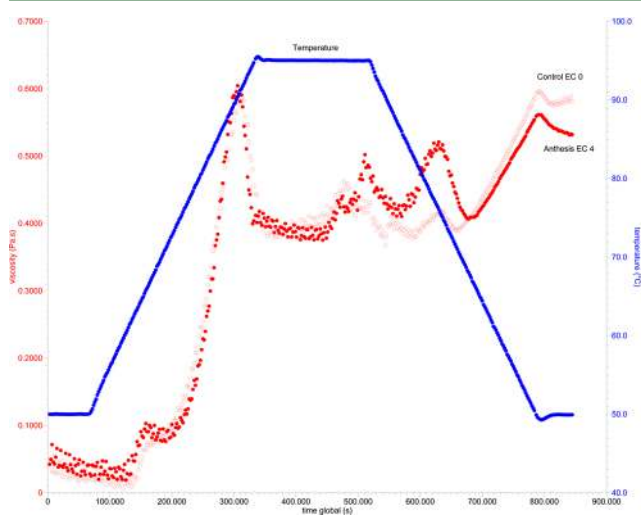

Figure 4. Pasting profiles of starches from Control and Anthesis 40 dS/m² NaCl-treated plants. An 8% (w/w) starch slurry was analyzed on the rheometer-simulated RVA platform. The temperature profile is shown in the figure.

deviate from that of the control sample. The same was shown in the study by Peiris et al.[7] with rice exposed to EC values ranging from 2 to 4 dS/m². The lower pasting temperature of the Anthesis EC4 starch may be explained by the diminished proportion of the long chains of amylopectin (Table 5), as these long chains could contribute to maintenance of starch granule integrity during the pasting process.[56] The peak viscosity and setback of most of the salt treatments were also lower than those of the control (Table 5), suggesting that the integrity of the gel network was compromised, thus reducing the occurrence of starch retrogradation.[60]

Multivariate Analysis. The PLS-DA algorithm is a supervised method that uses multiple linear regression analyses to identify maximum covariance between measured variables and treatments. In other words, the variation *within* groups is reduced in order to maximize differences *among* groups. The measured variables are summarized into a smaller number of variables called "scores" which then were plotted on the 2D-plots.[61] Here, PLS-DA was used to analyze the behavior of all variables measured in this study (Figure 5A), of the agronomic variables (Figure 5B), and of the grain composition and starch
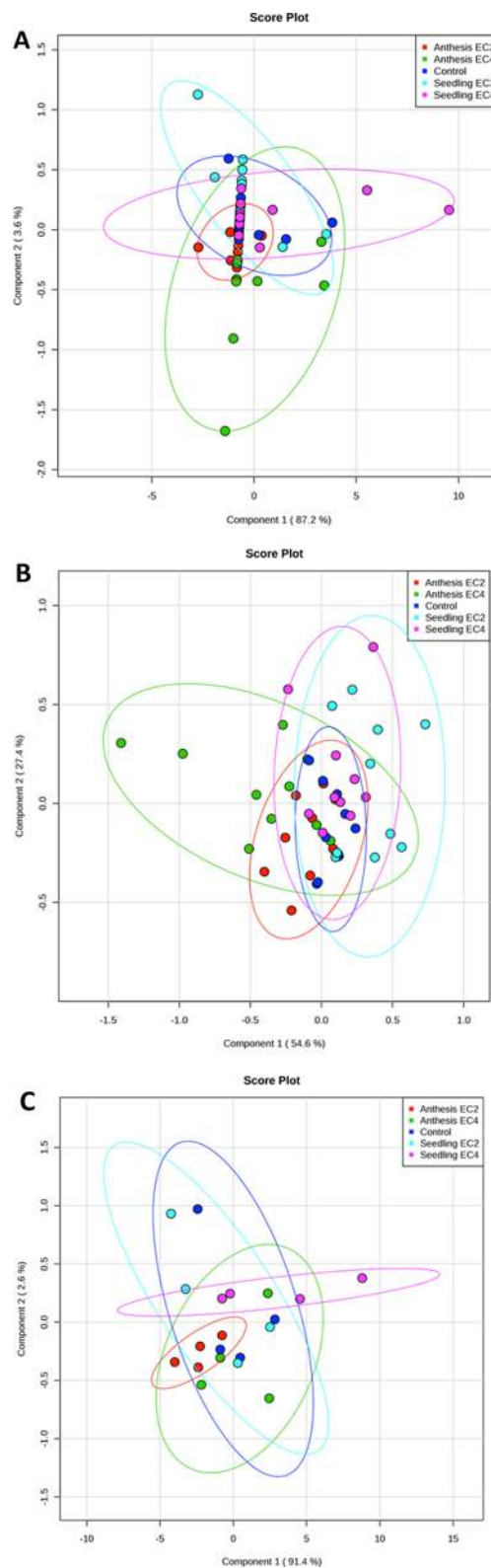

Figure 5. Partial-least squares discriminant analysis (PLS-DA) of measured variables. (A) PLS-DA plot of all variables in this study. (B) Agronomic yield and grain characteristic variables. (C) Grain composition and starch functionality variables. Each of the 3−10 replicates was individually plotted, and the samples were projected onto 2D-plots showing the first 2 principal components (PCs). Each PC captures maximum variation of the data set, where PC1 retains the most variation and PC2 captures the second most level of variation.[62] The confidence ellipses represent 95% confidence intervals of the data.

functionality variables (Figure 5C) in order to illustrate the separation between different groups of treatments. Although there were significant changes ($P < 0.05$) in the individual data sets of both agronomical and grain composition and starch functionality measurements, the multivariate analysis revealed that, when considered together, the variables behaved similarly among all the treatments as no distinct separation between treatment groups was observed (Figure 5A). Some separation could be seen between the Seedling EC2 Anthesis EC4 treatments when only the agronomical variables were taken into account (Figure 5B), but separation disappeared when grain composition and starch functionality parameters were simultaneously assessed (Figure 5C). Some separation between Anthesis EC2 and Seedling EC4 treatments however was observed when considering grain composition and starch functionality variables (Figure 5C). This implies that the impacts of different salinity levels applied at different developmental stages were not profound, at least in this greenhouse-based experimental setting in which other potential environmental variables were reduced.

This study aimed to understand and differentiate the prolonged and isolated effects at the grain filling stage of both mild and high salinity on the yield, and compositional and functionality components of rice grains. Overall, the changes were most pronounced in the high salinity treatment applied at the reproductive stages. Rice grown under both prolonged treatments seemed to have adapted to the salinity, but to different extents; that is, Seedling EC2 appeared to have morphological adaptation, while Seedling EC4 plants modulated their carbon flux to the developing kernels. Applying salt at Anthesis had a direct but opposite effect on grain filling process: Anthesis EC2 may have accelerated grain filling, resulting in higher starch accumulation, while Anthesis EC4 was compromised in its adaptability to the imposed salinity level, which was reflected in diminished agronomic yields and starch content. Although multivariate analysis of the data showed that the 2 and 4 dS/m^2 NaCl did not have profound effects on rice starch molecular composition regardless of the time of treatment application, there were still measurable changes in starch thermal and pasting properties, which were only limited to the most affected treatment (i e. Anthesis EC4). Overall, this study demonstrated the complexity of rice responses to timing and severity of salinity stress.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables S1, S2, and S3 and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ADP-glucose pyrophosphorylase, AGPase; degree of polymerization, DP; differential scanning calorimetry, DSC; electrical conductivity, EC; high-performance anion-exchange chromatography, HPAEC; nitrogen, N; partial-least squares discriminant analysis, PLS-DA; rapid viscosity analysis, RVA; sodium chloride, NaCl; sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on February 12, 2015, with an error in references 22, 33, 35, and 52. The corrected version reposted on February 17, 2015.