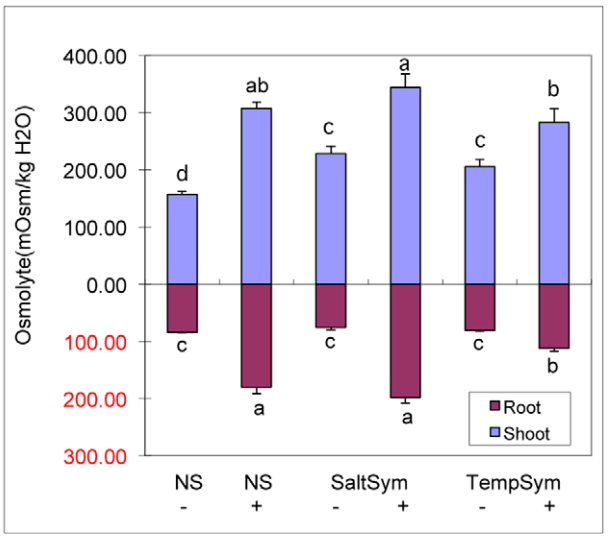
A



B

Paraquat-induced photobeaching in rice plants in the presence and absence of stress

Treatment	Salt Stress		Drought Stress	
	-	+	-	+
NS	1/9[a]	9/9[b]	0/9[c]	9/9[d]
SaltSym	0/9[a]	1/9[a]	0/9[c]	2/9[c]
TempSym1	ND	ND	0/9[c]	2/9[c]
TempSym2	ND	ND	0/9[c]	1/9[c]

Figure 5. Effect of symbiosis on plant osmolyte concentrations and paraquat-induced photobleaching (ROS) under laboratory conditions. A) Five week old rice plants (N = 30) that were NS or colonized with SaltSym or TempSym1 exposed for ten days in the absence (-) and presence (+) of salt stress (300 mM NaCl), at which point, the effects of stress began to show in NS plants treatments (≥70% wilted +/−chlorosis). SaltSym imparts salt tolerance and TempSym1 does not. Osmolyte concentrations (milliosmoles per kg wet weight) of roots and shoots were assessed and statistical analysis (Duncan's multiple-range test; SE≤9.98 &<23.73 for root, and shoot, respectively; P<0.0001 for root and shoot) indicated significantly higher levels in the shoots of S plants compared to NS plants in the absence of salt stress, and no statistical differences between treatments in the presence of salt stress. No significant differences were observed in roots in the absence of salt stress. In the presence of stress, Tempsym1+ showed significantly lower level of osmolytes than SaltSym+ and NS+ treatments. Values with the same letters are not significantly different. **B**) NS and S (SaltSym and TempSym1 & 2) plants exposed to salt (300 mM NaCl, 10 days) and drought stress (3 days) were tested for paraquat-induced photobleaching (ROS activity). Time points were chosen when symptoms began to appear (wilting and chlorosis) in NS stressed plants. Leaf disks (N = 9) from 9 independent plants were used for ROS assays. Leaf disks were sampled from leaf tissues of similar size, developmental age, and location for optimal side-by-side comparisons. Values indicate the number of leaf disks out of a total of nine that bleached white after exposure to paraquat indicating ROS generation. Statistical analysis (Duncan's multiple-range test) indicated that in the absence of stress, little to no (0–11%) photo beaching occurred in all the treatments. In contrast, significant differences occurred with 100% of the NS plant disks for both salt and drought stress bleaching white compared to only 11–22% of the S plant disks (P<0.0001). ND = not determined.
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salt stress, plants colonized with SaltSym showed no significant photobleaching (11%) while NS plant tissues bleached white (100%). Similarly, S plant tissues exposed to drought stress showed no significant photobleaching (11–22%) while NS plants did (100%).

Discussion

Rice plants were adapted to cold, salt and drought stress simply by colonization with Class 2 fungal endophytes. Salt and temperature stress tolerance are habitat-adapted traits of the endophytes evaluated in this study [33]. SaltSym, derived from coastal plants (*Leymus mollis)* exposed to high salt stress confer salt tolerance, and not temperature tolerance. TempSym1 & 2 were isolated from *Dichanthelium lanuginosum* thriving in geothermal soils conferred temperature tolerance and not salt tolerance. Since TempSym1 & 2 originated in geothermal soils differing in summer maximum and winter minimum temperatures, we anticipated that TempSym2 and not TempSym1 could confer cold tolerance. The fact that both endophytes conferred cold tolerance may reflect the cold winter temperatures plants experience above ground rather than in the soil.

Initial cell signaling and biochemical pathways involved in both hot and cold temperature stress responses begin with the same root physiological processes and later branch off into unique pathways [12]. It is tempting to speculate that TempSym1 & 2 regulate early events (as our ROS studies indicate) in the plant temperature response such that downstream responses do not occur resulting in tolerance to heat and cold.

To make observations concerning the impacts of cold stress on seedling shoot and root development, rice seeds showing a small white tissue protuberance (indicating a high potential success rate of germination) were used for the cold stress assays. Cold stress tolerance was conferred to germinated seeds under laboratory conditions by TempSym1 less than 24 hr post inoculation of seeds resulting in greater than 90% seedling development at temperatures between 5°–20°C (Fig.3). It is possible that TempSym1 either allows plants to increase metabolic rates at low temperatures or increase metabolic efficiency to overcome affects of low temperature. Symbiotically induced metabolic efficiency was also observed in laboratory studies showing decreased water consumption and increased biomass in S plants.

Salt and drought stress were tested under greenhouse and growth chamber conditions. SaltSym conferred salt tolerance, allowing plants to grow when continually exposed to a solution of 300 mM NaCl (Fig. 3). More importantly, a gradual increase in salt exposure of mature plants effectively eliminated seed production in NS plants. Although mature S plants had reduced seed production under salt stress compared to non-stressed plants, salt stressed S plants produced similar amounts of seed as NS plants grown in the absence of salt stress (Fig. 4). The number of stems of S plants was statistically higher (data not shown P≥0.05)) than NS plants which would prove to be beneficial to overall plant health and yields under field conditions. The levels of salt used in these studies are similar to those occurring in agricultural lands after tsunamis or tidal surges [37]. Therefore, we anticipate that using SaltSym may allow growers to mitigate the impacts of salt inundation.

A common physiological response to salt stress is an increase in the production of osmolytes [12]. In the absence of salt stress, osmolyte levels were similar in the roots of S and NS plants but significantly higher in the shoots of S plants compared to NS plants (Fig. 5). Upon exposure to salt stress both S and NS plants significantly increased osmolyte levels, but their responses differed with NS plants increasing by approximately 50% and symbiotic plants increasing approximately 30% compared to non-stressed plants. This was unexpected as previous results, albeit with a different stress, indicated that in the presence of heat stress,

osmolytes increased significantly in nonsymbiotic plants but either increased slightly or not at all in S plants [33]. These results suggest that osmolyte production in symbiotic plants varies either with the type of stress, endophyte genotype, and/or plant genotype. Regardless, since all plant treatments in this study responded in a similar manner (an overall increase in the presence of stress regardless of the treatment), it appears that osmolyte production alone is not responsible for symbiotic adaptation of rice plants to salt stress.

All three endophytes conferred drought tolerance to rice plants delaying wilt, 2–3 times beyond that of NS plants (Fig. 3). Although the mechanisms of endophyte conferred drought tolerance are not known, delayed wilt time correlated with a reduction of water usage (20–33%). Production of ROS is correlated with the early events in the plants stress response system. Our studies indicated that drought and salt tolerance in S plants correlated to decreased ROS activity (Fig. 5). All of the NS leaf tissues photobleached in the presence of paraquat while only 0–22% of the S plants showed any photobleaching. Increases in ROS are common to all stresses as a result of stress-induced metabolic imbalances [42,43]. The data suggest that in the presence of stress, either rice plants remain metabolically balanced or over express ROS scavenging antioxidation systems. Nevertheless, decreased ROS activity in S plants correlates strongly with stress tolerance and may play a critical role in the process.

The endophytes increased the potential fitness of rice plants by enhancing growth, development, biomass and yield in the presence and absence of stress as observed under laboratory and greenhouse conditions. The influence of the endophytes on plant growth and development was significant. Assessing percent biomass changes in the treatments revealed that there was a 6.59% decrease in shoot biomass in NS compared to SaltSym plants exposed to stress. In roots, the effects were more dramatic with 15.8% root biomass decrease in NS plant compared to SaltSym plants exposed to stress. These results suggest that SaltSym plants are able to better deal with the negative effects of salt stress than their NS counterparts. Although the basis of endophyte induced growth promotion is not known, the fungal endophytes are able to produce significant amounts ($\leq$500 ppm) of IAA, a plant growth hormone, when grown in culture (Table 2). Remarkably, enhanced growth was observed within 24 hr of colonization and observations indicated endophytes influenced the allocation of resources into roots and shoots (Fig. 2). Time-lapse imaging revealed that nonsymbiotic plants preferentially allocated resources into shoots prior to substantial root growth while S plants increased root mass prior to shoot growth. This is in contrast to plants grown under continual light: NS plants equally distributed resources into roots and shoots while the shoots of S plants had limited growth until root hairs were developed [44]. The difference in resource allocation in plants grown under continual light and a 14 hr light cycle indicates that under light conditions (12–14 hr) occurring during crop production, the impacts of symbiosis are much greater than under continual light. It is tempting to speculate that since symbiotic plants use less water, produce greater biomass, and have higher yields; the endophytes may allow rice plants to achieve greater metabolic efficiency.

Taxonomically, *L. mollis*, *D. lanuginosum* and rice are in the family (Poaceae) but are in different subfamilies (Pooideae, Panicoideae and Ehrhartoideae, respectively). Previous studies indicated that SaltSym and TempSym1 could also colonize and confer habitat-specific stress tolerances to the eudicot tomato [27,33] suggesting that the symbiotic communication responsible for stress tolerance is conserved among plant lineages. The ability of endophytes to colonize and confer stress tolerance, increase yields and biomass, and disease resistance [28] to genetically unrelated plant species suggests that they may be useful in adapting plants to drought, salt and temperature stresses that are predicted to worsen in future years due to climate change.

Materials and Methods

Assessing endophyte colonization from plant tissues

At the beginning and end of each experiment, the efficiency of endophyte colonization of inoculated plants and the absence of endophytes in mock inoculated controls was assessed as follows: a subset of at least 10% of laboratory and greenhouse plants were assessed for colonization. Plants were washed until soil debris was removed, placed in to plastic zip-loc baggies and surface sterilized as previously described [28,45]. Using aseptic technique, plants were cut into sections representing the roots and stem sections, imprinted [46], plated on fungal growth media (see below), and incubated 5–7 days at 22°C with a 12 hr light cycle (cool fluorescent lights) to allow for the emergence of fungi. Upon emergence, fungal endophytes were identified using microbiological and molecular techniques as previously described [33]. The effectiveness of surface sterilization was verified using the imprint technique [46].

Fungal cultures. *Fusarium culmorum* and *Curvularia protuberata* species (Table 1) were cultured on 0.1X potato dextrose agar (PDA) medium supplemented with 50–100 μg/ml of ampicillin, tetracycline, and streptomycin, and fungal cultures grown at 22°C with a 12 hr light regime. After 5–14 days of growth, conidia were harvested from plates by adding 10 ml of sterile water and gently scraping off spores with a sterile glass slide. The final volume of spores was adjusted to 100 ml with sterile water, filtered through four layers of sterile cotton cheesecloth gauze and spore concentration adjusted to 10^3–10^4 spores/ml.

Rice Varieties. *Oryza sativa*, var. M-206 (subspecies Japonica) and Dongjin (subspecies Indica) were collectively used in greenhouse and laboratory studies. M-206 is a variety predominantly grown in Northern California and Dongjin in South Korea.

Plant colonization. For laboratory and greenhouse studies, seeds were surface sterilized in 2.5% (v/v) sodium hypochlorite for 24–48 hr, rinsed with 5–10 volumes of sterile distilled water, and imbibed in 1–2 volumes of water for 8–12 hr. Seeds were germinated on 1% agar water medium, maintained at 26°–30°C and exposed to a 12 hr fluorescent light-regime. To ensure that our studies began only with nonsymbiotic plants, seedlings that showed no outgrowth of fungi into the surrounding media were chosen and transplanted. Any seedlings showing outgrowth of fungi, were discarded. Endophyte-free plants (up to 20 plants/magenta box depending upon the study) were planted into sterile double-decker magenta boxes (modified magenta boxes to hold soil or sand in upper chamber, and fluid in lower chamber that is wicked-up through a cotton rope; [33]) containing equivalent amounts (380+/−5 g) of sterile-sand, or Sunshine Mix #4 [Steuber Distributing Co., WA, USA (40+/−0.5 g)]. The lower chamber was filled with 200 ml of sterile water or 1x Hoagland's solution supplemented with 5 mM $CaCl_2$. After 1 week, plants were either mock inoculated with water (nonsymbiotic) or inoculated with fungal endophytes by pipetting 10–100 ul of spores (10^3–10^4/ml) at the base of the crowns or stems. Plants were grown under a 12 hr light regime at 26°–30°C for 3–5 weeks for laboratory, and 2 months for greenhouse studies prior to imposing stress.

Growth response and development. Symbiotically induced growth response of root and shoot development was visualized in seedlings through time-lapse photography. Nonsymbiotic (NS) and symbiotic (S) seeds were generated by placing surface sterilized

seeds on agar plates (containing 1x Hoagland's solution supplemented with 5 mM $CaCl_2$) and flooding the plates with water (mock-inoculated) or fungal spores (10^2–10^3/ml) for 48 hr. Three geminated seedlings were chosen at random from a total of N = 100 seeds. The representative seedlings were placed onto a vertical plant growth apparatus for photographic monitoring. The apparatus was comprised of two 3 mm thick glass plates (30 cm×30 cm), with a divider (high pressure tubing) placed down the center, and the whole apparatus sealed around the edges using Tygon tubing and clamps to generate two separated leak-free compartments. One hundred fifty ml of 1x Hoagland's media (supplemented with 5 mM $CaCl_2$ and 1% agarose) was poured into each compartment and allowed to solidify. A thin layer (2–3 mm) of silica sand was placed on top of the solid matrix. All components and media used were either surface sterilized in 70% EtOH or autoclaved prior to assembly. Three germinated seedlings were then embedded in the sand on each side of the compartment. Seedlings were maintained at 26°C in a 14 hour light/dark regime using daylight balanced fluorescent studio lights. Photos were taken every 20 min using a Canon PowerShot G5 camera and a Pclix infrared Controller (Toronto, Canada).

Abiotic stresses. Greenhouse and laboratory experiments were performed with plants grown in magenta boxes at 26°–30°C in a temperature controlled room with a 12 hr fluorescent light regime. Magenta boxes were randomly placed in different locations on shelves in the growth room for salt and drought stress experiments. Each experiment was repeated three times and the images in the figures are representative of all replications of each treatment.

Magenta boxes contained<20 plants and the total number of plants/replication is indicated as (N = XX) in the figure legends. The health of plants was assessed on a scale of 1–5 (1 = dead, 2 = severely wilted and chlorotic, 3 = wilted +/− chlorosis, 4 = slightly wilted, 5 = healthy w/o lesions or wilting), and is listed in the figure legends.

Control plants – All control plants were maintained at 26–30°C and hydrated throughout the experiment with sterile water or 1x Hoagland's solution supplemented with 5 mM $CaCl_2$.

Salt – plants were exposed to 100–300 mM NaCl in 1x Hoagland's solution supplemented with 5 mM $CaCl_2$ (referred to as 100 mM, 200 mM and 300 mM NaCl solution) for 10–21 days for laboratory studies by filling the lower chamber of the double-decker magenta boxes with 200 ml of the salt solutions. Greenhouse studies with mature plants were exposed to 100 mM–300 mM NaCl for up to 3 months (see below). After plants were showing symptoms (i.e., nonsymbiotic plants dead or severely wilted), they were re-hydrated in sterile water devoid of NaCl for 48 hr, plant health assessed and photographed. All laboratory assays were repeated three times.

Drought – watering was terminated for 3–15 days by decanting off the fluid in the lower chamber of the double-decker magenta box and letting the plant soils dry out over-time. A hydrometer (Stevens Vitel Inc.) was used to ensure that soil moisture levels were equivalent between treatments when watering was terminated. After plants showed symptoms (i.e., NS plants dead or severely wilted), they were re-hydrated in sterile water for 48 hr, plant health assessed and photographed. All assays were repeated three times.

Plant water usage and biomass. Water consumption was measured in double-decker magenta boxes. Initially, 200 ml of 1x Hoagland's solution supplemented with 5 mM $CaCl_2$ were placed in the lower chamber. Fluid remaining in the lower chamber after 10 days of plant growth was measured, and water usage calculated as ml consumed/10 days. All assays were repeated three times.

Cold Stress. The effect of cold stress was assessed using surface sterilized and imbibed seeds (see plant colonization above for details) that were either mock-water or fungal spore (10^2–10^3/ml) inoculated by immersion in solutions for 48 hr with gentle agitation. Twenty NS and S seedlings (seeds exhibiting a small white tissue radical - indicative of germination) were placed on solid water agar (1%) plates and incubated at 5°C, 10°C, 15°C, and 20°C, and the % seedling development assessed after 10 days. All assays were repeated three times.

Salt stress versus plant biomass and yields. The effect of symbiosis on the growth and yield of mature rice plants exposed to prolonged salt stress (3 months) was tested with NS and S (endophyte conferring salt tolerance referred to as SaltSym)] plants under greenhouse conditions. Plants were generated using standard protocols (as described above). Two-month old plants were either maintained as control non-stressed plants and watered with 1x Hoagland's solution supplemented with 5 mM $CaCl_2$ or salt stressed plants exposed to 100 mM–300 mM NaCl in 1x Hoagland's solution supplemented with 5 mM $CaCl_2$. Stressed plants were initially exposed to 100 mM NaCl solution for 3 weeks, exposed to 200 mM NaCl solution for an additional 3 weeks, and then exposed to 300 mM NaCl solution until the termination of the studies (approximately 6 weeks for a total of 5 months for the study). Upon termination of the studies, the spikes were removed and weights determined (g). Prior to plant biomass assessment (wet weight), roots and shoots were gently washed to remove dirt and debris. Roots and stems were than blotted dry, cut into root and shoot sections, and weights determined (g). Representative photos were taken of all treatments.

Colony Forming Units (CFU). NS and S plants were surface sterilized (described above) and 5 plants (total of 0.5 g) pooled to obtain equal amounts of roots and lower stems. Plant tissues were homogenized (Tekmar tissue homogenizer) in 10 ml of STC osmotic buffer (1M Sorbitol, 10 mM TRIS-HCl, 50 mM $CaCl_2$, pH 7.5) on ice and 100 µl plated onto 0.1XPDA fungal growth medium (see above). After 5–7 days at 25°C, CFU were assessed. All assays were repeated three times.

Plant osmolyte concentrations. NS and S plants exposed to +/− salt stress were analyzed for osmolyte concentrations. Equivalent amounts of root and lower stem tissues (100 mg total) from 3–5 plants/condition were ground in 500 ul water with 3 mg sterile sand, boiled for 30 min, samples cooled to 25°C, centrifuged for 5 min at 6 K rpm, and osmolytes measured with a Micro Osmometer 3300 (Advanced Instruments). All assays were repeated three times.

Reactive Oxygen Species (ROS). NS and S plants were exposed to +/− salt (300 mM NaCl solution) and drought stress for 3–10 days and leaf tissue samples taken just prior to or when slight to moderate stress induced symptoms were observed. Using a cork borer, leaf discs (2 mm) were obtained from each of 3 replicate plants from different magenta boxes and placed on a solution of 1 uM of the herbicide paraquat (N,N′-Dimethyl–4,4′-bipyridinium dichloride, Syngenta, Greensboro, NC) and incubated at 22°C under fluorescent lights. After 48 hr exposure to paraquat, leaf discs were photographed to document chlorophyll oxidation visualized by tissue bleaching. All assays were repeated three times.

IAA Assays. *Fusarium* sp. and *Curvularia* sp. were cultured on 0.02-0.1X potato dextrose agar (PDA) medium and 0.2-1X modified Mathur's media [MS [47]]. Both media were supplemented with 50–100 ug/ml of ampicillin, tetracycline, and streptomycin. Indole-3-acetic acid (IAA) assays of mycelia grown in liquid media was assessed by growth on 0.1X PDA or 1X MS media as follows: 3–5 plugs of fungal mycelia plugs were

inoculated into liquid media and grown for 2 days at 22°C with agitation (220 rpm). After 2 days, mycelia were blended (Tekmar) and grown an additional 1–2 days until a dense suspension of mycelia was achieved. Mycelia were filter through 4 layers of sterile gauze and washed with 10 volumes of sterile water. Ten percent mycelia (g/100 ml) was inoculated into 0.2X MS liquid media +/−0.1% tryptophan (Trp) and grown with agitation (220 rpm) for up to 3 weeks. One ml of supernatant was collected at various time points (24 hr–21 days), the supernatant passed through spin columns (Millipore centrifugal filter units) to remove mycelia and pigment, and IAA assessed using Fe-$HClO_4$ solution colorimetric assay (OD = 530 nm) protocol [41] and levels compared to an IAA (Sigma) standard curve of 0–1000 ppm. IAA levels in NS and S [*F. culmorum* isolate FcRed1 (SaltSym) and *C. protuberata* isolate Cp4666D (TempSym1)] 5 day old rice seedlings were assessed by grinding x10 plants (seeds removed) in liquid N_2, re-suspending plant debris in an equal volume of sterile water, vortexed 1 min, incubated for 30 min at 22°C, and the supernatant passed through spin columns and assessed for IAA as described above. IAA standards were passed through spin columns with no hindrance. All assays were conducted three times.

Statistical analysis. P values were determined by Duncan's multiple-range test and data analyzed using SAS [48].

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Author Contributions

Conceived and designed the experiments: RSR RJR. Performed the experiments: RSR YOK CJDAW CG LE. Analyzed the data: RSR YOK CJDAW CG LE RJR. Contributed reagents/materials/analysis tools: RSR YOK CG LE SLD RJR. Wrote the paper: RSR RJR.

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