

## ORIGINAL ARTICLE

# Stress tolerance in plants via habitat-adapted symbiosis

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**We demonstrate that native grass species from coastal and geothermal habitats require symbiotic fungal endophytes for salt and heat tolerance, respectively. Symbiotically conferred stress tolerance is a habitat-specific phenomenon with geothermal endophytes conferring heat but not salt tolerance, and coastal endophytes conferring salt but not heat tolerance. The same fungal species isolated from plants in habitats devoid of salt or heat stress did not confer these stress tolerances. Moreover, fungal endophytes from agricultural crops conferred disease resistance and not salt or heat tolerance. We define habitat-specific, symbiotically-conferred stress tolerance as habitat-adapted symbiosis and hypothesize that it is responsible for the establishment of plants in high-stress habitats. The agricultural, coastal and geothermal plant endophytes also colonized tomato (a model eudicot) and conferred disease, salt and heat tolerance, respectively. In addition, the coastal plant endophyte colonized rice (a model monocot) and conferred salt tolerance. These endophytes have a broad host range encompassing both monocots and eudicots. Interestingly, the endophytes also conferred drought tolerance to plants regardless of the habitat of origin. Abiotic stress tolerance correlated either with a decrease in water consumption or reactive oxygen sensitivity/generation but not to increased osmolyte production. The ability of fungal endophytes to confer stress tolerance to plants may provide a novel strategy for mitigating the impacts of global climate change on agricultural and native plant communities.**

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## Introduction

Plant responses to abiotic stresses (for example, salinity, heat and drought) are complex involving signal reception and transduction followed by genetic and physiological responses. All plants are thought capable of perceiving and responding to stress (Bohnert *et al.*, 1995; Bartels and Sunkar, 2005). Plant responses common to these stresses include osmolyte production, altering water transport and scavenging reactive oxygen species (ROS) (Leone *et al.*, 2003; Maggio *et al.*, 2003; Tuberosa *et al.*, 2003). Still, relatively few species are able to thrive in habitats that impose high levels of abiotic stress (Alpert, 2000). Despite extensive research in plant stress responses (Smallwood *et al.*, 1999), two questions remain unanswered: What are the mechanisms by which plants adapt to abiotic stress?

Why is there not a greater diversity and ecological distribution of stress-tolerant plants?

Fossil records indicate that fungi have been associated with plants since at least 400 MYA (Redecker *et al.*, 2000; Krings *et al.*, 2007) and fungal symbiosis is thought responsible for the movement of plants onto land (Pirozynski and Malloch, 1975). There are at least three classes of fungal symbionts: the well-studied mycorrhizal fungi and class 1 endophytes and the lesser defined class 2 endophytes (Rodriguez *et al.*, 2005), which were the focus of this study. A great deal is known about mycorrhizal fungi that are found associated with plant roots and share nutrients with their plant hosts, and about the clavicipitaceous fastidious endophytes (class 1) that infect cool season grasses (Read, 1999; Schardl *et al.*, 2004). Recently, the ecological roles of class 2 endophytes have begun to be elucidated (Redman *et al.*, 2002; Arnold *et al.*, 2003; Rodriguez *et al.*, 2005; Waller *et al.*, 2005; Schulz, 2006). Class 2 endophytes have a broad host range of both monocot and eudicot plants, are the largest group of fungal symbionts, are readily culturable on artificial media and are thought to colonize all plants in natural ecosystems (Petrini,

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1996). Although the occurrence of endophytes have been extensively studied since the 1970s, there has been no comprehensive analysis of a plant community (Carroll, 1988). Assessing species richness and diversity of class 2 endophytes in plant communities is a daunting task as these endophytes can be numerous and represent undescribed species as studies with the western white pine have shown (Ganley *et al.*, 2004). Ganley *et al.* hypothesize that 'If endophytes are generally unknown species, then estimates of 1 million endophytes (that is, approximately 1 in 14 of all species of life) seem reasonable.'

Studies have shown that class 2 endophytes confer stress tolerance to host species and play a significant role in the survival of at least some plants in high-stress environments (Rodriguez *et al.*, 2004). For example, class 2 endophytes confer heat tolerance to plants growing in geothermal soils (Redman *et al.*, 2002), the extent of tree leaf colonization by endophytes correlates with the ability to resist pathogens (Arnold *et al.*, 2003) and endophytes confer drought tolerance to multiple host species (Waller *et al.*, 2005). On the basis of laboratory and field studies of class 2 endophytes from plants from geothermal soils, coastal beaches and agricultural fields, we describe a newly observed ecological phenomenon defined as habitat-adapted symbiosis. Utilizing Koch's postulates, we have determined that endophytes from these habitats confer habitat-specific stress tolerance to plants. This habitat-specific phenomenon provides an intergenomic epigenetic mechanism for plant adaptation and survival in high-stress habitats.

## Materials and methods

### *Recovering endophytes from plant tissues*

Between the spring of 2000 and summer of 2003, *Leymus mollis* (dunegrass ( $N=200$ )) plants were collected from several coastal beach habitats in the San Juan Island Archipelago, WA. Plants were washed until soil debris was removed, placed into plastic zip-loc baggies and surface sterilized as previously described (Redman *et al.*, 2001, 2002a). Using aseptic technique, plants were cut into sections representing the roots, rhizomes and stems, plated on fungal growth media (see below) and incubated at room temperature for 5–7 days under cool fluorescent lights to allow for the emergence of fungi. Upon emergence, 30 representative isolates of the dominant fungal endophyte represented ( $\geq 95\%$ ) were sub-cultured and, of these, single-spore isolation of 10 representative isolates was performed as previously described (Redman *et al.*, 1999). All 10 of the representative isolates were placed under sterile water supplemented with 50–100  $\mu\text{g ml}^{-1}$  of ampicillin in sterile 1.5 ml screw-cap tubes and placed at 4 °C for long-term storage. Of the 10 representative isolates, three were randomly chosen for species identification (described below).

The effectiveness of surface sterilization was verified using the imprint technique (Schulz *et al.*, 1999).

### *Fungal cultures*

*Fusarium* and *Curvularia* species were cultured on 0.1 × potato dextrose agar (PDA) medium and the *Colletotrichum magna* isolate L2.5 was cultured on a modified Mather's media (MS; Tu, 1985). Both media were supplemented with 50–100  $\mu\text{g ml}^{-1}$  of ampicillin, tetracycline and streptomycin, and fungal cultures grown at 22 °C with a 12-h 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  $10^4$ – $10^5$  spores  $\text{ml}^{-1}$ .

### *Fungal identification*

Fungi were identified using conidiophore and conidial morphology (Arx, 1981; Barnett and Hunter, 1998; Leslie and Summerell, 2005). Once the original 10 single-spored isolates from *L. mollis* were identified as the same fungal species microscopically, three of the isolates were randomly selected for molecular species identification. Species designations were based on sequence analysis of the variable ITS1 and ITS2 sequences of rDNA (ITS4 (5'-tcctccgcttattgatatgc-3')/ITS5 (5'-ggaagtaaaagtcgtaacaagg-3') primers) and translation elongation factor (EF1T (5'-atgggtaaggaggacaagac-3')/EF2T (5'-ggaagta ccagtgatcatgtt-3') and EF11 (5'-gtggggcattacccgcc-3')/EF22 (5'-aggaaccttaccgagctc-3') primers (White *et al.*, 1990; O'Donnell *et al.*, 2000)). DNA was extracted from mycelia and PCR amplified as previously described (Rodriguez and Yoder, 1991; Rodriguez, 1993; Redman *et al.*, 2002a). PCR products were sequenced and BLAST searched against the GenBank database. Morphological and GenBank analysis identified the three isolates/species as the same species (*Fusarium culmorum*) at which point, one isolate designated FcRed1 (for *F. culmorum* red-pigmented isolate no. 1) was chosen for the studies presented here. An isolate of *F. culmorum* (Fc18) was purchased from American Type Culture Collection for sequence comparison.

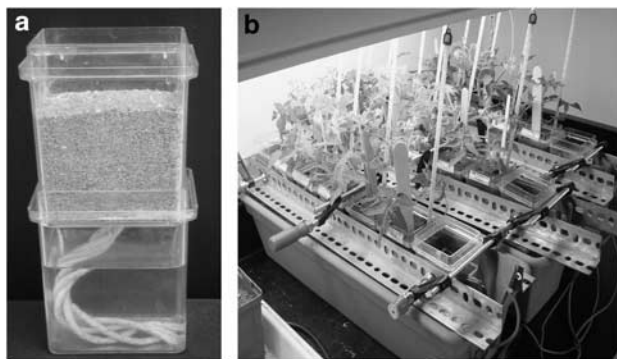
### *Plant colonization*

Tomato (*Solanum lycopersicum* var. Tiger-Like), dunegrass (*L. mollis*) and panic grass (*Dichanthelium lanuginosum*) seeds were surface sterilized in 0.5–1.0% (v/v) sodium hypochlorite and/or 1% silver nitrate solution for 15–20 min with moderate agitation and rinsed with 10–20 volumes of sterile distilled water. Rice (*Oryza sativa* subspecies

*Japonica*, var. Dongjin) seeds were surface sterilized in 70% ethanol for 30 min, then transferred to 5% (v/v) sodium hypochlorite for 30 min with moderate agitation and rinsed with 10–20 volumes of sterile distilled water. Plant seeds were germinated on 1% agar medium supplemented with 1 × Hoagland's solution or 0.1 × PDA medium, maintained at 22–28 °C and exposed to a 12-h fluorescent light regime. To ensure that our studies began only with non-symbiotic plants, seedling that showed no outgrowth of fungi into the surrounding medium were chosen and transplanted. Any seedlings showing outgrowth of fungi were discarded.

Endophyte-free plants (up to 30 plants/magenta box depending upon the plant species) were planted into sterile double-decker magenta boxes containing equivalent amounts (380 ± 5 g) of sterile sand or Sunshine Mix no. 4 (40 ± 0.5 g; Figure 1). The lower chamber was filled with 200 ml of sterile water or 1 × Hoagland's solution supplemented with 5 mM CaCl<sub>2</sub>. After 1–4 weeks, plants were either mock-inoculated with water (nonsymbiotic) or inoculated with fungal endophytes by pipetting 10–1000 µl of spores (10<sup>4</sup>–10<sup>5</sup> ml<sup>-1</sup>) at the base of the crowns or stems. Plants were grown under a 12-h light regime at 22–28 °C (depending on the plant species) for 1–4 weeks prior to imposing stress.

At the beginning and end of each stress experiment, the efficiency of endophyte colonization of inoculated plants and the absence of endophytes in



**Figure 1** (a) Modified magenta box constructed by drilling a hole at the base of the upper magenta box, top-knotting and weaving through a defined length of cotton rope to the bottom chamber to act as a wick and adding a defined amount of sand or soil in the upper chamber. Fluid is added to the bottom chamber and a tight-fitting lid is added to the top (not shown) and the whole system autoclaved and sterilized prior to symbiotic or nonsymbiotic plant transplantation. (b) Geothermal soil stimulator. The top half of the modified magenta box containing the plant is removed and wrapped with thermal tape at the soil or sand line and temperature regulated by a Thermolyne rheostat controller (Barnstead International, Dubuque, IA, USA). Utilizing thermal tape, the geothermal stimulators were designed by this research team such that the soil/root zone is exposed to elevated temperature to mimic what occurs in the natural geothermal habitat. Modified magenta boxes were secured together using a system of clamps and metal brackets and the entire assemblage (with exposed dangling cotton wicks) placed into tubs containing copious amounts of water. A thermometer was placed in each magenta box to monitor temperature accurately throughout the experiment.

mock-inoculated controls was assessed as follows: a subset of plants representing 10–30% of each treatment were surface sterilized, cut into sections (roots, stem or crown, and leaf), imprinted (Schulz *et al.*, 1999) and placed on 0.1 × PDA medium to assess fungal colonization as previously described (Redman *et al.*, 1999, 2001, 2002a). After 5–7 days of growth at 22 °C with a 12-h light regime, fungi growing out of plant tissues were identified using standard taxonomic techniques as described above.

#### Abiotic stresses

Experiments were performed with plants grown in magenta boxes at 22–28 °C (depending on the plant species) in a temperature-controlled room with a 12-h fluorescent light regime. Magenta boxes were randomly placed in different locations on shelves in the growth room for salt and drought stress experiments. Plants used in heat stress experiments (panic grass and tomato) were randomly placed in geothermal soil simulators (Figure 1; Redman *et al.*, 2002). The root zones were exposed to ± heat for 12 h increments to mimic the soils that are hottest during the day and cool down at night as previously demonstrated (Redman *et al.*, 2002). Each experiment was repeated a minimum of three times, and the images in the figures are representative of all replications of each treatment.

Magenta boxes contained 1–30 plants (depending upon the plant species and assay) 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 with out lesions or wilting), and is listed in the figure legends.

**Control plants:** all control plants were maintained at room temperature (22 °C) and hydrated throughout the experiment with sterile water or 1 × Hoagland's solution supplemented with 5 mM CaCl<sub>2</sub>.

**Salt:** tomato, rice and dunegrass plants were exposed to 300–500 mM NaCl in 1 × Hoagland's solution supplemented with 5 mM CaCl<sub>2</sub> (referred to as 300 or 500 mM NaCl solution) for 10–14 days by filling the lower chamber of the double-decker magenta boxes with 200 ml of one of these salt solutions. After plants started showing symptoms (that is, nonsymbiotic plants dead or severely wilted), they were re-hydrated in sterile water devoid of NaCl for 24–48 h, plant health assessed and photographed. All assays were repeated a minimum of three times.

**Drought:** watering was terminated for 7–14 days (depending on the plant species) by decanting off the fluid in the lower chamber of the double-decker magenta box and letting the plant soils dry out overtime. A hydrometer (Stevens-Vitel Inc., Chantilly, VA, USA) was used to ensure that soil moisture levels were equivalent between treatments when watering was terminated. After plants started

showing symptoms (that is, nonsymbiotic plants dead or severely wilted), they were re-hydrated in sterile water for 24–48 h, plant health assessed and photographed. All assays were repeated a minimum of three times.

**Heat:** tomato seedlings or 3- to 12-week-old tomato and panic grass plants were placed in geothermal soil simulators (Figure 1) and root zones heat stressed by ramping up temperatures from ambient to 50 °C in 5-°C increments every 24–48 h. The first symptoms of heat stress were observed after 5 days in tomato seedlings and 10–12 days in larger tomato and panic grass plants. Tomato seedling plant health was assessed and photographed after 5 days of heat stress and experiments continued for an additional 48 h, at which point, only Cp4666D symbiotic plants survived. All assays were repeated a minimum of three times.

#### Field study

Commercially available *L. mollis* seeds were surface sterilized and used to generate symbiotic ((with FcRed1 ( $N=20$ )) and nonsymbiotic ( $N=20$ ) plants as described above. Plants were grown in sterile potting soil (Sunshine Mix no. 4) for 3 months in a cold frame greenhouse exposed to ambient temperature and light. A replicate set of plants (20/treatment) was used to ensure that nonsymbiotic plants were free of fungi and that symbiotic plants contained FcRed1 as described above. Plants were transplanted into a beach habitat located at the University of Washington's Cedar Rocks Preserve (CR-SJI; Shaw Island, WA, USA). Three months after transplanting, plants were removed with root systems intact and transported back to the laboratory to assess plant viability and biomass (described below), prevalence of ecto-mycorrhizal associations (visual observations) and class 2 fungal colonization (described above).

#### Plant water usage and biomass

Water consumption was measured in rice, tomato, panic grass and dunegrass plants in double-decker magenta boxes. Initially, 200 ml of 1 × Hoagland's solution supplemented with 5 mM CaCl<sub>2</sub> was placed in the lower chamber. Fluid remaining in the lower chamber after 5 days of plant growth was measured and water usage calculated as ml consumed/5 days. Plant biomass was measured by gentle removal of plants from magenta boxes followed by rinsing roots with water to remove soil debris, blotting on paper towels to remove excess water and weighing individual plants. All assays were repeated a minimum of three times.

#### Colony-forming units

Symbiotic and nonsymbiotic plants were surface sterilized (described above) and five plants (total of

0.5 g) pooled to obtain equal amounts of roots and lower stems. Plant tissues were homogenized (Tekmar tissue homogenizer, Vernon, British Columbia, Canada) in 10 ml of STC osmotic buffer (1 M Sorbitol, 10 mM TRIS-HCl, 50 mM CaCl<sub>2</sub> (pH 7.5)) on ice and 100 µl plated onto 0.1 × PDA fungal growth medium (see above). After 5–7 days at 22 °C, colony-forming units (CFU) were assessed. All assays were repeated a minimum of three times.

#### Assessing the rhizosphere fungal community

Approximately 300 g of substrate (ranging from fine sand and soil to coarse small pebbles) surrounding dunegrass plants growing in the beach habitat was collected and processed within 24 h for fungal CFU analysis. Coarse pebbles larger than 1 mm in diameter were sifted out and the remaining sand/soil mixed and 3 g of soil placed in 30 ml of sterile water, vortexed for 30 s and 100 µl of a 10E–0 and 10–E1 dilution plated onto 0.1 × PDA medium. After 5–7 days at 22 °C, CFU were assessed. All assays were repeated a minimum of three times.

#### Axenic fungal growth and temperature and salt sensitivity

Growth of Cp4666D versus CpMH206 and FcRed1 versus Fc18 were compared for sensitivity to temperature and salt, respectively. Small plugs (1 mm) of mycelia were obtained from cultures growing on 0.1 × PDA plates using a cork borer and placed in the center of 100 mm plates containing (1) 0.1 × PDA and placed at 25–40 °C for the temperature sensitivity assay; or (2) either H<sub>2</sub>O Agar or 1 × PDA ± 500 mM NaCl for the salt sensitivity assay. Colony diameters were measured at a minimum of three positions every 24 h for 5–6 days and growth rates averaged from three independent plates. Growth was recorded as mm/24 h and standard deviations for all assays were ≤ 3 mm.

#### Plant osmolyte concentrations

Nonsymbiotic and symbiotic plants exposed to ± temperature stress were analyzed for osmolyte concentrations. Equivalent amounts of root and lower-stem tissues (100 mg total) from three plants/condition were ground in 500 µl water with 3 mg sterile sand, boiled for 30 min, samples cooled to 25 °C, centrifuged for 5 min at 6K r.p.m. and osmolytes measured with a Micro Osmometer 3300 (Advanced Instruments, Norwood, MA, USA). All assays were repeated a minimum of three times.

#### Reactive oxygen species (ROS)

Symbiotic and nonsymbiotic plants were exposed to ± heat (50 °C for 12 h) and salt (300 mM NaCl solution) stress for 5–7 days and leaf-tissue samples taken just prior to or when slight-to-moderate symptoms were observed. Using a cork

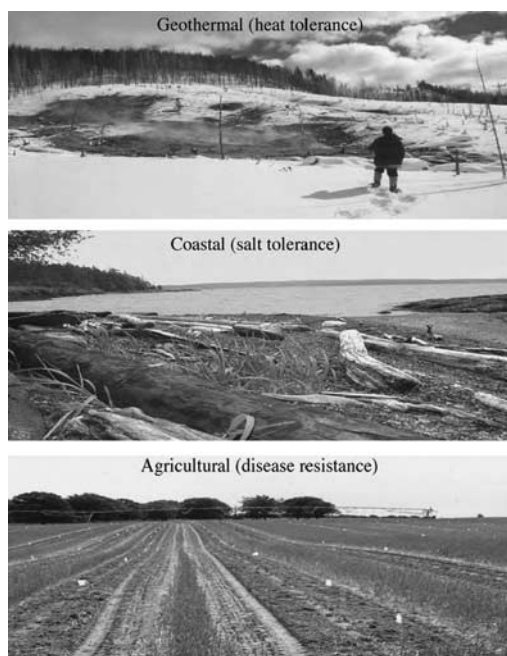
borer, four leaf (3–5 mm) disks were obtained from each of the three replicate plants from different magenta boxes and placed on a solution of 1  $\mu\text{M}$  of the herbicide paraquat (*N,N*-dimethyl-4,4'-bipyridinium dichloride, Sygenta Basal, Wilmington, DE, USA) and incubated at 22 °C under fluorescent lights. After 24–48 h exposure to paraquat, leaf disks were photographed to document chlorophyll oxidation visualized by tissue bleaching. All assays were repeated a minimum of three times.

#### Statistical analysis

*P*-values were determined by analysis of variance single-factor analysis and data analyzed using SAS (SAS Institute, 2000).

### Coastal habitat

Plant communities on Puget Sound beaches of Washington State are commonly dominated by *L. mollis*. In this habitat, plants are exposed to sea water during high tides and summer seasons are typically very dry. These plants are rhizome-forming perennial grass species that can achieve high population densities and remain green until they senesce in the fall (Imbert and Houle, 2000). Two hundred dunegrass individuals, all growing in similar beach habitats (course rocky soils, Figure 2), were collected from four geographically distant locations ( $\geq 16$  km) in Puget Sound and found to be colonized by one dominant class 2 fungal

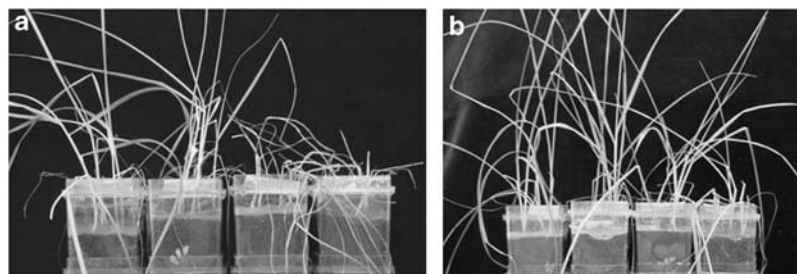


**Figure 2** Three unique field habitats addressed in our studies. Each imposing very different stresses: geothermal soil of YNP where the habitat-specific stress is high soil temperatures (top panel); coastal beach regions of CR-SJI where the habitat-specific stress is salt stress (middle panel); and agricultural arena where the habitat-specific stress is high disease pressure (lower panel).

endophyte, which was isolated from surface sterilized roots, rhizomes, crowns and lower stems in 95% of the plants analyzed. The endophyte was also isolated from seed coats and upper leaf sections with lower frequency (11–70%), but were not isolated from seeds. The dominant *L. mollis* endophyte was identified as *F. culmorum* by microscopic and DNA sequence analysis (BLAST analysis indicated a 100% similarity with *F. culmorum* sequences for rDNA ITS1, ITS2 and elongation factor). Other filamentous fungi were isolated from *L. mollis* at frequencies  $\leq 5\%$  and were not included in these studies. The surface sterilization technique is very stringent and eliminates epiphytic fungi, thereby allowing for assessment of class 2 endophyte colonization, which are culturable on artificial media (Schulz *et al.*, 1999; Rodriguez *et al.*, 2005). However, due to the stringent nature of the surface sterilization technique, some samples contained no fungi (that is, 10 of the original 200 dunegrass plants were oversterilized and devoid of fungi). To better understand the distribution of the *L. mollis* endophyte in the coastal habitat, rhizosphere soils were analyzed for the presence of filamentous fungi. Although there was a high abundance of filamentous fungi ( $> 1\text{E}5$  CFU/g soil) in the rhizosphere, *F. culmorum* was present at very low densities ( $< 0.05\%$  of culturable fungi).

On the basis of the abiotic stresses imposed in the coastal habitats, we tested the ability of *F. culmorum* (isolate FcRed1) to confer salt and drought tolerance to dunegrass plants under laboratory conditions (Figure 3). In the absence of stress, there were no observable differences in the development and health of nonsymbiotic and symbiotic plants but there were significant increases in symbiotic plant biomass (average biomass of 60 plants =  $1.31 \text{ g} \pm 0.23$  (symbiotic) and  $0.97 \text{ g} \pm 0.03$  (nonsymbiotic);  $P = 0.061$ ). However, with constant exposure to 500 mM NaCl solution (sea water levels to mimic exposure of plants in their native beach habitat), nonsymbiotic plants became severely wilted and desiccated within 7 days and were dead after 14 days. In contrast, symbiotic plants did not show wilting symptoms until they were exposed to 500 mM NaCl solution for 14 days (Figure 3a). The first signs of the effects of salt stress on symbiotic plants are seen as a slight curling and desiccation of the leaf tissue when compared to non-stressed control plants, which remained green and hydrated. Although some impact from 500 mM NaCl is apparent in FcRed1 symbiotic plants, the effects of this stress are much less when compared to nonsymbiotic plants. Thus, FcRed1 confers salt tolerance to levels equivalent to that of sea water.

To ensure that symbiotic plants maintained their endophytes throughout the experiment and verify that nonsymbiotic plants did not have endophytes associated with them, fungal colonization was assessed prior to and after stress was imposed for each experiment (performed for all subsequent



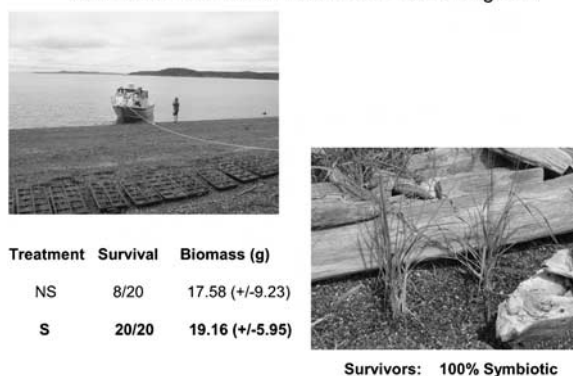
**Figure 3** Effect of symbiosis on salt and drought tolerance on native dunegrass (monocot) plants under laboratory conditions. The number of plants/treatment are indicated by ( $N=XX$ ), and the % survival and health of surviving plants are indicated in parentheses after each treatment. Plant health was based on comparison to nonsymbiotic controls and rated from 1 to 5 (1 = dead, 2 = severely wilted and chlorotic, 3 = wilted  $\pm$  chlorosis, 4 = slightly wilted, 5 = healthy with out lesions or wilting). All assays described from left to right and images are representative of all plants/treatment. (a) Dunegrass plants ( $N=30$ ), non-stressed controls (representative of both symbiotic and nonsymbiotic plants), symbiotic with FcRed1 (100%, 5), symbiotic with Fc18 (0%, 1) or nonsymbiotic (0%, 1) exposed to 500 mM NaCl for 14 days. While all plants bent over with age, unstressed controls and salt-exposed FcRed1 colonized plants remained hydrated while the other treatments wilted and lost turgor. (b) Dunegrass plants ( $N=30$ ), non-stressed controls (representative of both symbiotic and nonsymbiotic plants), symbiotic with FcRed1 (100%, 4), symbiotic with Fc18 (100%, 4) or nonsymbiotic (0%, 1) grown without water for 14 days.

studies as well). In all the cases, no fungi emerged from mock-inoculated plants (0% colonization) and all inoculated plants had the fungus they were inoculated with emerge from their tissues (100% colonization).

The ability of FcRed1 to confer drought tolerance was determined by the length of time required for symbiotic and nonsymbiotic plants to wilt after watering was terminated. Dunegrass plants colonized with FcRed1 wilted after 14 days without water while nonsymbiotic plants wilted after 6 days and were dead after 14 days (Figure 3b).

A field study was performed to determine if FcRed1 was required for survival of dunegrass plants in coastal habitats. Symbiotic and nonsymbiotic plants were transplanted as two independent clusters to a coastal beach habitat in the Cedar Rocks Preserve where dunegrass resides, is exposed to sea water at high tide and is known to harbor FcRed1 as an endophyte. Prior to transplanting,  $\pm$  colonization of symbiotic and nonsymbiotic plants, respectively, was verified. Three months after transplanting, the plants were evaluated for survival and biomass (Figure 4). All (100%) of the dunegrass plants initially colonized with FcRed1 survived, but only 40% of the nonsymbiotic plants survived in this coastal beach habitat. The average biomass of surviving symbiotic and nonsymbiotic plants was not statistically significant ( $P=0.607$ ), and when analyzed, the surviving nonsymbiotic plants were found to be colonized with *F. culmorum* suggesting that these plants were colonized in the field sometime after planting. To determine that the *F. culmorum* present in the originally nonsymbiotic field plants was indeed FcRed1, the endophytes were re-isolated, tested for symbiotic function and found to confer salt tolerance under laboratory conditions equivalent to that demonstrated in Figure 3a. Therefore, we surmised that the survival and final biomass of nonsymbiotic plants were dependent on the timing of *in situ* colonization by

2006 San Juan Island Field Trials with Dunegrass



**Figure 4** 2006 field experiment in a coastal beach habitat of CR-SJI with symbiotic (FcRed1) and nonsymbiotic (NS) generated dunegrass plants. Two clusters of plants (10 plants/cluster) were planted in late spring and assessed for survival, plant biomass and endophyte colonization 3 months later (shown in photograph). Statistical analysis revealed that there were significant differences in survival in symbiotic versus nonsymbiotic plants ( $P=4.59 \text{ E}-06$ ). While there were surviving plants in the NS treatment, microbiological analysis revealed that these plants were colonized with FcRed1.

FcRed1. This suggests that although FcRed1 is present in very low levels in soils, it is very efficient at colonizing *L. mollis*. Interestingly, roots of all plants were colonized with mycorrhizal fungi regardless of survival indicating that either mycorrhizal associations are not required for salt tolerance or that salt tolerance requires a combination of FcRed1 and mycorrhizal symbioses (that is, non-surviving plants had mycorrhizae but not FcRed1 while all surviving plants had both associations). Future studies need to be conducted addressing the potential synergistic roles of FcRed1 and mycorrhizal fungi in this three-way symbiosis.

*F. culmorum* is known as a cosmopolitan pathogen of monocots and eudicots (Farr *et al.*, 1989); however, FcRed1 asymptotically colonized

species from both plant groups (Table 1). Remarkably, FcRed1 conferred salt tolerance to rice (monocot) and tomato (eudicot) indicating that the association between FcRed1 and dunegrass was not a tight co-evolutionary relationship with regard to stress tolerance (Table 1; Figure 5a and c).

As previously observed with a class 2 endophyte from geothermal plants (Redman *et al.*, 2002;

**Table 1** Host colonization and stress tolerance conferred by fungal endophytes

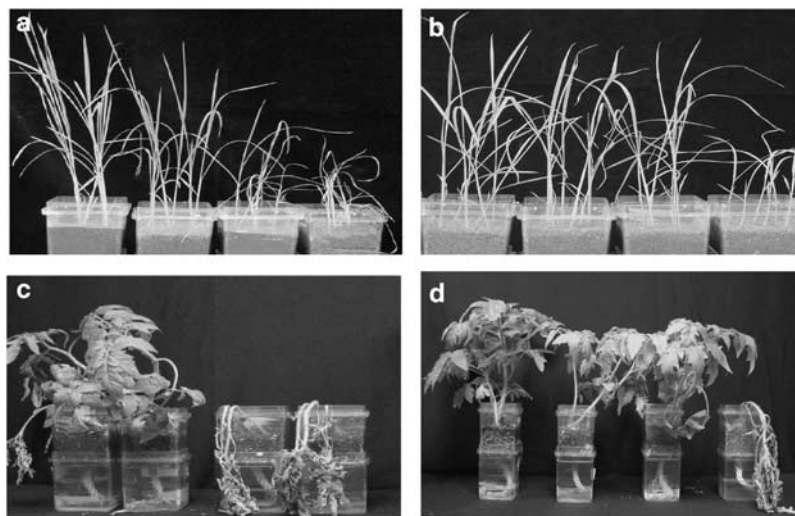
Endophyte	Dunegrass	Panic grass	Rice	Tomato
Cp4666D	r, s, D	r, s, D, H	r, s, D	r, s, D, H
CpMH206	ND	r, s, D,	ND	r, s, D,
FcRed1	r, s, D, S	r, s, D, S	r, s, D, S	r, s, D, S
Fc18	r, s, D,	ND	r, s, D,	r, s, D,

Abbreviation: ND, not determined.

Plant colonization ( $N=5$ ) was assessed by surface sterilization, cutting plants into root (r) and stem (s) sections and plating sections on fungal growth medium and surface sterilization verified using the imprint technique (Schulz *et al.*, 1999). Plant sections are listed only if fungi that grew out from those tissues. Symbiotically conferred drought and heat tolerance was assessed and denoted as D or H, respectively. Drought and heat tolerance was assessed after 7–14 days. Salt tolerance (S) was assessed by watering plants with 300–500 mM NaCl solution for 10–14 days. Stress tolerance was assessed as plant health and rated from 1 to 5 (dead and healthy, respectively; see Materials and methods). The % survival and health of stress-tolerant plants was 100% rated 4–5, and 100% rated 1 for stress-intolerant plants.

Henson *et al.*, 2005), microscopic analysis revealed that FcRed1 was able to grow on the surface of hosts and inside plants by intercellular growth with no indication of cellular disruption (not shown). This growth pattern appears to have no negative impacts on plant growth and fitness as indicated by the field experiment (Figure 4).

To determine if salt tolerance was unique to FcRed1 and other cohorts from dunegrass, we obtained *F. culmorum* isolate Fc18 from the American Type Culture Collection. Fc18 was isolated from an agricultural habitat in the Netherlands that does not impose salt stress. Both isolates asymptotically colonized dunegrass, rice and tomato plants; however, only FcRed1 conferred salt tolerance to these plant species (Table 1; Figures 3a, 5a and c). This suggests that FcRed1-conferred salt tolerance is a habitat-specific symbiotically adapted phenomenon. It is possible that the inability of Fc18 to confer salt tolerance was based on insufficient host colonization, an inability to confer fitness benefits or fungal sensitivity to salt. Colonization studies revealed that in the absence of stress, FcRed1 and Fc18 colonized hosts equivalently ( $P=0.43-0.68$ ). In contrast, in the presence of salt stress, CFU analysis indicated that FcRed1 was present in higher quantities than Fc18 in dunegrass plants (Table 2). Additional studies indicated that both FcRed1 and Fc18 conferred similar levels of



**Figure 5** Effect of symbiosis on salt and drought tolerance in the model rice (monocot) and tomato (eudicot) under laboratory conditions. The number of plants/treatment are indicated by ( $N=XX$ ), and the % survival and health of surviving plants are indicated in parentheses after each treatment. Plant health was based on comparison to nonsymbiotic controls and rated from 1 to 5 (1 = dead, 2 = severely wilted and chlorotic, 3 = wilted  $\pm$  chlorosis, 4 = slightly wilted, 5 = healthy with out lesions or wilting). All assays described from left to right and images are representative of all plants/treatment. (a) Rice plants ( $N=120$ ), non-stressed controls (representative of both symbiotic and nonsymbiotic plants), symbiotic with FcRed1 (100%, 5), symbiotic with Fc18 (0%, 1) or nonsymbiotic (0%, 1) exposed to 500 mM NaCl for 10 days. While all plants bent over with age, unstressed controls and salt-exposed FcRed1 colonized plants remained hydrated while the other treatments wilted and lost turgor. (b) Rice plants ( $N=120$ ), non-stressed controls (representative of both symbiotic and nonsymbiotic plants), symbiotic with FcRed1 (100%, 5), symbiotic with Fc18 (100%, 5) or nonsymbiotic (0%, 1) grown without water for 10 days. (c) Tomato plants ( $N=12$ ), non-stressed controls (representative of both symbiotic and nonsymbiotic plants), symbiotic with FcRed1 (100%, 5), symbiotic with Fc18 (0%, 1) or nonsymbiotic (0%, 1) exposed to 300 mM NaCl for 14 days. (d) Tomato plants ( $N=12$ ), non-stressed controls (representative of both symbiotic and nonsymbiotic plants), symbiotic with FcRed1 (100%, 5), symbiotic with Fc18 (100%, 5) or nonsymbiotic (0%, 1) grown without water for 10 days.

**Table 2** Effects of heat and salt stress on fungal colonization of plants

Fungal isolate	CFU			
	Dunegrass +Salt stress	Panic grass +Heat stress	Tomato +Salt stress	Tomato +Heat stress
Cp4666D	ND	11.0±4.0 (0.048)	ND	4.3±1.5 (0.067)
CpMH206	ND	3.7±2.2 (0.048)	ND	1.0±1.7 (0.067)
FcRed1	4.8±1.64 (0.027)	ND	5.6±1.15 (0.001)	ND
Fc18	2.4±1.14 (0.028)	ND	1.4±1.14 (0.001)	ND

Abbreviations: ANOVA, analysis of variance; CFU, colony-forming units; ND, not determined.

Monocot (panic grass) and eudicot (model tomato) plants that were colonized by Cp4666D that imparts temperature tolerance and CpMH206 that does not were exposed to temperature stress (50 °C for 12 h, 22 °C for 12 h for 12 days) and CFU assessed. Similarly, monocot (dunegrass) and eudicot (model tomato) plants that were colonized with FcRed1 that imparts salt tolerance and Fc18 that does not were exposed to salt stress (300 mM NaCl solution for 14 days) and CFU assessed. Equal amounts of plant tissues were processed for CFU analysis. Standard deviations are on the right of the ± sign and *P*-values were determined by ANOVA single-factor analysis and are in parentheses.

drought tolerance (Table 1; Figures 3b, 5b and d) indicating that both endophytes were able to establish communication with the plant and confer fitness benefits. These results were not surprising as our earlier studies indicated that different isolates of the same fungal species could express different lifestyles (that is, pathogenic versus mutualistic) depending upon the plant host they were interacting with (Redman *et al.*, 2001). Moreover, comparison of ITS sequences and fungal morphology indicated that Fc18 and FcRed1 are the same species, although functionally (conferring salt tolerance to plants) different. Therefore, we conclude that the salt tolerance conferred by FcRed1 is a habitat-specific symbiotic adaptation.

Saprophytic growth of *F. culmorum* was assessed by measuring growth rates of FcRed1 and Fc18 on artificial media varying in nutrient content ± NaCl equivalent to sea water levels (Table 3). Both isolates grew on minimal (H<sub>2</sub>O water) medium with no significant differences in growth rates in the absence of salt (*P* = 0.67). However, in the presence of NaCl, Fc18 grew much faster than FcRed1 on minimal medium (*P* = 1.88E−04). On rich medium, Fc18 grew significantly faster than FcRed1 in the absence (*P* = 3.0E−03) and presence (*P* = 1.75E−05) of NaCl. Interestingly, the growth rate of FcRed1 was significantly less in the presence of salt on both media (*P* = 0.024–0.065). In contrast, Fc18 grew significantly faster in minimal medium in the presence of NaCl (*P* = 0.021) and no significant differences observed in rich medium ± NaCl (*P* = 0.78). These results were intriguing as isolate Fc18, which does not impart salt tolerance to plants, yet is able to grow faster in the presence of salt (Table 3). Salt sensitivity may explain why FcRed1 is not abundant in the rhizosphere soils since the soils are inundated with sea water during high tides. The high abundance of FcRed1 in *L. mollis* and the low abundance of this isolate in the rhizosphere suggest that FcRed1 may not be very competitive in the rhizosphere and survives more successfully as an endophyte within the plant host.

**Table 3** Saprophytic growth rates (mm per 24 h) of fungal isolates ± stress

Isolates	H <sub>2</sub> O agar medium		1 × PDA medium	
	−Salt	+Salt	−Salt	+Salt
FcRed1	1.36±0.09	1.08±0.25	1.48±0.13	1.26±0.15
Fc18	1.14±0.11	1.70±0.45	1.74±0.22	2.28±0.28
	1 × PDA medium			
	25 °C	30 °C	37 °C	40 °C
Cp4666D	25.33±2.29	28.00±1.58	6.89±1.38	NG
CpMH206	28.78±2.49	40.89±5.25	8.67±2.24	NG

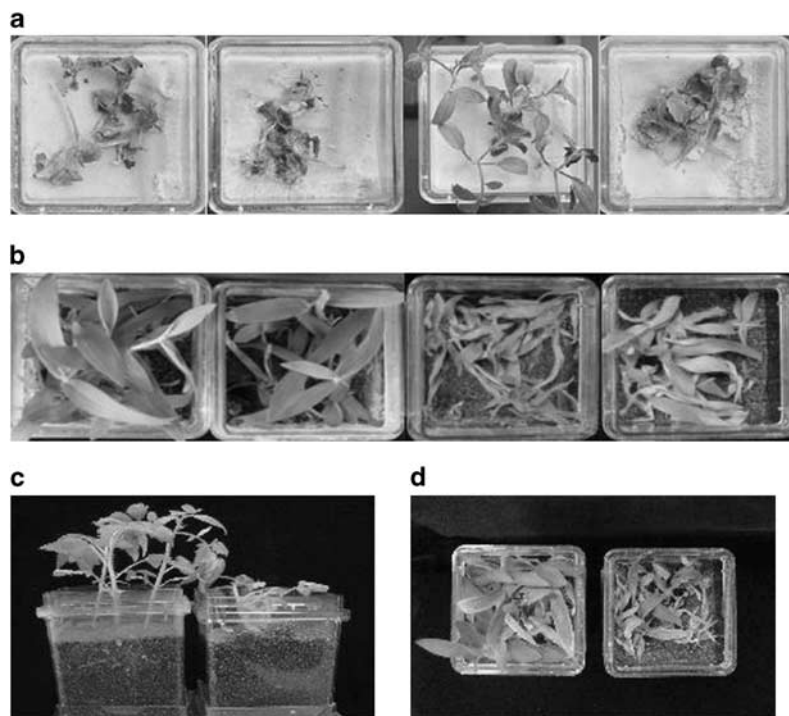
Abbreviations: NG, no growth; PDA, potato dextrose agar.

Salt stress: isolates were grown at 25 °C on different media ± 500 mM NaCl; temperature stress: isolates were grown on one medium at 25–40 °C.

## Geothermal soil habitat

We previously reported that a fungal endophyte (*Curvularia protuberata*) was responsible for thermotolerance (over a 12-month field trial and under laboratory conditions) of the monocot *Dichanthelium lanuginosum* (panic grass), which thrives in geothermal soils of Yellowstone National Park, Wyoming (YNP) (Figure 2 (Redman *et al.*, 2002; Márquez *et al.*, 2007)). Moreover, neither the plant nor the endophyte survived temperatures above 38 °C indicating that this association was a mutualism with both partners achieving survival under field conditions (Redman *et al.*, 2002). Here, we present data comparing symbiotically conferred benefits of *Curvularia protuberata* from panic grass in YNP (isolate Cp4666D) and an isolate of *Curvularia protuberata* (CpMH206) obtained from American Type Culture Collection that originated from a grass (*Deschampsia flexuosa*) growing in a non-geothermal habitat in Scotland, UK. Molecular comparison of these isolates revealed that they share 100% similarity in the rDNA ITS1, ITS2 and





**Figure 6** Effect of symbiosis on heat (**a** and **b**) and drought tolerance (**c** and **d**) on the genetic model tomato (**a** and **c**) and native panic grass (**b** and **d**) under laboratory conditions. The number of plants/treatment are indicated by ( $N=XX$ ), and the % survival and health of surviving plants are indicated in parentheses after each treatment. Plant health was based on comparison to nonsymbiotic controls and rated from 1 to 5 (1 = dead, 2 = severely wilted and chlorotic, 3 = wilted  $\pm$  chlorosis, 4 = slightly wilted, 5 = healthy with out lesions or wilting). All assays described from left to right and images are representative of all plants/treatment. (**a**) Tomato seedlings ( $N=30$ ) symbiotic with FcRed1 (0%, 1), CpMH206 (0%, 1) or Cp4666D (100%, 5), or nonsymbiotic (0%, 1) exposed to 50 °C root temperatures for 5 days. Although not shown, non-stressed plants (representative of both symbiotic and nonsymbiotic plants) remained green and healthy throughout the experiment. (**b**) Panic grass ( $N=30$ ), non-stressed controls (representative of both symbiotic and nonsymbiotic plants), symbiotic with Cp4666D (100%, 5), symbiotic with CpMH206 (0%, 1) or nonsymbiotic (0%, 1) exposed to 50 °C root temperatures for 12 days. (**c**) Tomato plants ( $N=30$ ) symbiotic with CpMH206 (100%, 5), or nonsymbiotic (0%, 1) grown without water for 7 days. (**d**) Panic grass ( $N=30$ ) symbiotic with CpMH206 (85%, 5; 15% 3), or nonsymbiotic (0%, 1) grown without water for 7 days. Although not shown, non-stressed controls (representative of both symbiotic and nonsymbiotic plants) remained hydrated and healthy (100%, 5) as did drought-stressed Cp4666D (100%, 5) in both tomato and panic grass (**c** and **d**).

elongation factor sequences. Colonization studies indicated that Cp4666D and CpMH206 equally colonized tomato and panic grass in the absence of stress ( $P=0.24-0.74$ ). However, when exposed to heat stress (Figures 6a and b), the CFU of Cp4666D increased and that of CpMH206 decreased in both tomato and panic grass (Table 2). While Cp4666D conferred heat tolerance and hence plant survival to both tomato and panic grass plants, CpMH206 did not and is reflected in the overall CFU. To determine if the inability to confer temperature tolerance was based on sensitivity to temperature, axenic growth of CpMH206 and Cp4666D exposed to 25–40 °C was compared (Table 3). Interestingly, the growth rate of Cp4666D was significantly less than CpMH206 at 25 °C, 30 °C and 37 °C ( $P=0.008-0.059$ ). No growth of either isolate was observed above 40 °C. These studies indicate that Cp4666D is more sensitive to temperature outside of the plant, which may explain why Cp4666D is not abundant in the geothermal rhizosphere soils (<0.001%).

Although CpMH206 did not impart heat tolerance, it was of interest to determine if this isolate could confer any fitness benefits. To determine if

heat tolerance was an isolate-specific, habitat-adapted phenomenon, drought studies were performed (Table 1; Figures 6c and d). Both Cp4666D and CpMH206 conferred similar levels of drought tolerance in tomato and dunegrass indicating that CpMH206 was capable of conferring fitness benefits to plant hosts.

### Agricultural habitats

Our previous studies were centered around the biochemical and molecular basis of plant disease caused by pathogenic *Colletotrichum* species (Freeman and Rodriguez, 1993; Redman *et al.*, 2001, 1999, 2002a). A host-range study of eight *Colletotrichum* species revealed that these virulent plant pathogens have the ability to express non-pathogenic lifestyles depending upon the hosts they colonize. For example, *Colletotrichum magna* isolate CmL2.5 is a virulent pathogen of cucurbits but asymptotically colonizes tomato (Redman *et al.*, 2001). Depending on the tomato genotype, CmL2.5 increases plant growth rates and/or fruit yields, and

confers drought tolerance and/or disease resistance against virulent pathogens (Redman *et al.*, 2001). Experiments performed during these studies revealed that *Colletotrichum* species do not confer salt or heat tolerance to tomato or cucurbits and the *Curvularia* and *Fusarium* isolates (Cp4666D and FcRed1, respectively) do not confer disease resistance (not shown). Therefore, *Colletotrichum* species are adapted to agricultural habitat-specific stresses (high disease pressure) and confer disease resistance to plant hosts, another example of habitat-adapted symbiosis (Figure 2). As seen with the *Curvularia* and *Fusarium* isolates described above, the *Colletotrichum* species also confer drought tolerance (Redman *et al.*, 2001).

## Defining symbioses

There are several outcomes of symbiotic interactions defined by the fitness benefits realized by each partner (Lewis, 1985). Benefits to fungal symbionts can be positive (mutualism, commensalism and parasitism), neutral (amensalism and neutralism) or negative (competition). Benefits to host plants can also be positive (mutualism), neutral (commensalism and neutralism) or negative (parasitism, competition and amensalism). While it is fairly straightforward to determine the impact of symbiosis on host fitness, it is more challenging to determine the benefits for fungal endophytes. Mutualistic benefits for endophytes may involve acquiring nutrients from hosts, abiotic and biotic stress avoidance and dissemination by seed transmission (Schardl *et al.*, 2004; Schulz, 2006). The fact that the associations reported here resulted in positive host fitness benefits (stress tolerance and growth enhancement) suggests that the symbioses are mutualisms. The following aspects of endophyte biology also suggest that these associations are mutualisms: (1) Cp4666D (Redman *et al.*, 2002) and FcRed1 are vertically transmitted via the colonization of seed coats; (2) both endophytes are in high abundance in plants and low abundance in rhizosphere soils suggesting that the fungi are not competitive in the rhizosphere; (3) fungal growth in plants requires a nutrient source that must be generated by the host; (4) Cp4666D does not survive above 40 °C but can be isolated from plant roots heated to 65 °C indicating successful stress avoidance; and (5) growth of FcRed1 is negatively impacted by high levels of NaCl suggesting that it avoids salt stress via symbiosis.

## Stress tolerance mechanism(s)

Interestingly, all of the endophytes in this study conferred drought tolerance to monocot and eudicot hosts regardless of the habitat of origin. Studies indicate that plant–fungal symbiosis might have been in existence for  $\geq 400$  MYA (Krings *et al.*,

2007), and might have played an important role in the movement of plants from an aquatic arena to comparatively dry terrestrial habitats (Pirozynski and Malloch, 1975). When this happened, plants must have been confronted with intermittent water stress. It is tempting to speculate that the ability to confer drought tolerance may simply be a legacy shared by all endophytes.

Drought, heat and salt stress affect plant–water relations triggering complex plant responses, which include increased production of osmolytes (Bohnert *et al.*, 1995; Bray, 1997; Wang *et al.*, 2003). Osmotic potential is determined primarily by two components: solute potential and matrix potential, and it is likely that symbiotic fungi contribute to the matrix potential, which is particularly important in helping plants retain water and thereby enhance plant drought tolerance. Upon exposure to heat stress, nonsymbiotic panic grass and tomato plants significantly increased osmolyte concentrations as predicted. Increased osmolyte concentrations, not surprisingly, correlated with the development of subsequent wilting and desiccation symptoms prior to plant death. In contrast, symbiotic plants either maintained the same (panic grass and Rutgers tomato (Márquez *et al.*, 2007)) or lower (Tiger-Like tomato (Table 4)) osmolyte concentrations when compared to non-stressed controls. The differences in osmolyte patterns in tomato may be reflective of differences in the varieties (Rutgers versus Tiger-Like). Regardless, the overall pattern of osmotic concentrations in plants that succumb to heat stress (nonsymbiotic) differs from plants that are heat-stress tolerant, suggesting that symbiotic plants use approaches other than increasing osmolyte concentrations to mitigate the impacts of heat stress.

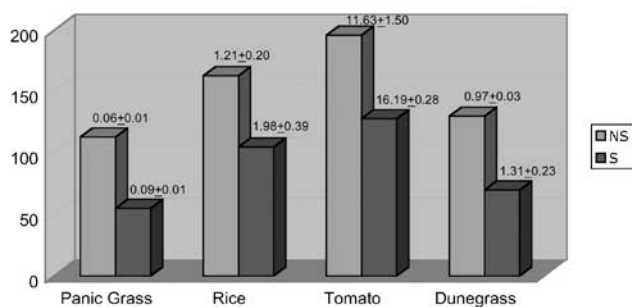
Symbiotic plants consumed significantly less water than nonsymbiotic plants regardless of the colonizing endophyte. Figure 7 shows representative profiles of symbiotic versus nonsymbiotic plant fluid uptake. Panic grass, rice, tomato and dunegrass plants all used significantly less fluid than nonsymbiotic plants ( $P = \leq 0.04$ ). Since these symbiotic plants achieve increased biomass levels ( $P = 0.29$ – $0.061$ ; discussed above), decreased water consumption suggests more efficient water usage. Decreased

**Table 4** Effect of symbiosis on plant osmolyte concentrations

Treatment	Without stress		With heat stress	
	Panic grass	Tomato	Panic grass	Tomato
NS	57 ± 5.1 <sup>a</sup>	178 ± 8.7 <sup>b</sup>	142 ± 13.2 <sup>c</sup>	263 ± 24.7 <sup>c</sup>
S	102 ± 7.2 <sup>b</sup>	206 ± 15.6 <sup>b</sup>	114 ± 5.7 <sup>b</sup>	127 ± 34.7 <sup>a</sup>

Nonsymbiotic (NS) and symbiotic (S, with Cp4666D) plants were maintained at 22 °C (–stress) or with root zones heated to 50 °C for 12 days (+stress) and osmolyte concentrations (milliosmole per kg wet wt) ± s.d. values assessed. Assays were repeated a minimum of three times. Values with the same letters are not significantly different (Duncan's multiple-range test,  $P < 0.0005$ ).

**Fluid Usage ml/5days in Symbiotic vs. Nonsymbiotic Plants**



**Figure 7** Water usage in symbiotic (S) and nonsymbiotic (NS) plants ( $N=25, 120, 30$  and  $60$  for panic grass, rice, tomato and dunegrass, respectively) was quantified over time and expressed as fluid consumed (ml)/5 days with s.d. values no greater than 12.5 ml. Statistical analysis revealed significant differences in fluid usage ( $P \leq 0.04$ ) and biomass ( $P=0.013-0.061$ ) with symbiotic plants using less fluid and having increased biomass (numerical value above each bar = average weight (g)  $\pm$  s.d. of three representative plants from each treatment) compared to nonsymbiotic plants.

water consumption and increased water-use efficiency may provide a unique mechanism for symbiotically conferred drought tolerance.

One plant biochemical process common to all abiotic and biotic stresses is the accumulation of ROS. Generation of ROS is an early event in plant response to stress (Apel and Hirt, 2004; Rodriguez *et al.*, 2005). ROS are extremely toxic to biological cells causing oxidative damage to DNA, lipids and proteins. One way to mimic endogenous production and assess tissue tolerance to ROS is to expose photosynthetic tissue to the herbicide paraquat, which is subsequently reduced by electron transfer from plant photosystem I and oxidized by molecular oxygen resulting in the generation of superoxide ions and subsequent photobleaching (Vaughn and Duke, 1983). We exposed symbiotic and nonsymbiotic plants to  $\pm$  stress (panic grass and tomato to heat stress, and dunegrass and tomato to salt stress) and exposed excised plant leaf disks to paraquat (Table 5). In the absence of stress, both nonsymbiotic and symbiotic plant leaf tissues for all plants (panic grass, tomato, dunegrass) remained green indicating the absence of ROS generation, and hence lack of stress response. In contrast, when exposed to stress, nonsymbiotic tissues bleached white indicating the generation of ROS while symbiotic tissues remained green. This suggests that endophytes either scavenge ROS, induces plants to more efficiently scavenge ROS or prevents ROS production when exposed to abiotic stress.

Class 1 and class 2 fungal endophytes differ in several aspects: class 1 endophytes comprise a relatively small number of fastidious species that have a few monocot hosts and class 2 endophytes (described here) comprise a large number of tractable species with broad host ranges including both monocots and eudicots. In addition, the role of

**Table 5** Effect of symbiosis on ROS generation in the presence or absence of stress

Plant	Treatment	-Heat stress	+Heat stress	-Salt stress	+Salt stress
Panicgrass	NS	0/12	12/12	ND	ND
	S	0/12	0/12	ND	ND
Tomato	NS	0/12	11/12	0/12	10/12
	S	0/12	0/12	0/12	1/12
Dunegrass	NS	ND	ND	1/12	11/12
	S	ND	ND	0/12	1/12

Abbreviations: ND, not determined; ROS, reactive oxygen species. Monocot (panic grass and dunegrass) and eudicot (model tomato) plants that were symbiotically (S) colonized with either Cp4666D or FcRed1 (that imparts heat and salt tolerance, respectively) compared to nonsymbiotic (NS) plants were exposed to  $\pm$  temperature or salt stress (see text for details) and assayed for ROS. Leaf disks ( $N=12$ ) were excised from  $N=3$  plants/treatment. The values indicate the number of leaf discs out of a total of 12 that bleached white after exposure to paraquat indicating ROS generation.

ROS in plant symbioses with class 1 and class 2 endophytes may differ. The class 1 endophyte *Epichloe festucae* appears to generate ROS to limit host colonization and maintain mutualisms (Tanaka *et al.*, 2006), while the class 2 endophytes Cp4666D and FcRed1 reduce ROS production to possibly mitigate the impact of abiotic stress. Additional studies will indicate if these are general differences between class 1 and class 2 endophytes or a reflection of individual isolates.

## Habitat-adapted symbiosis

The ability of endophytes originally isolated from grasses to confer the same functional stress tolerance to genetically distant plants such as tomato is intriguing as the evolutionary divergence of these plants occurred approximately 140–235 MYA (Wolfe *et al.*, 1989; Yang *et al.*, 1999; Chaw *et al.*, 2004). Moreover, the concept that fungal endophytes adapt to stress in a habitat-specific manner was confirmed with different fungal and plant species, and different environmental stresses. We define this phenomenon as habitat-adapted symbiosis and hypothesize that fungal endophytes provide an intergenomic epigenetic mechanism for plant adaptation to habitat stresses. In fact, our field studies in YNP (Redman *et al.*, 2002) and Puget Sound (Figure 4) indicated that habitat-adapted symbiosis can result in stress tolerance of plants within a single growing season. While it is clear that class 2 fungal endophytes provide a mechanism for plant adaptation, this does not explain why so few plants are adapted to high-stress habitats. Since class 2 endophytes appear to have broad host ranges and can confer stress tolerance to genetically distant plant species (at least under controlled laboratory conditions), one would expect that a greater number of plants would adapt to high-stress environments.

Clearly, fungal endophytes are only part of the story. Nevertheless, we hypothesize that class 2 endophytes play an important ecological role in the distribution patterns of plants and therefore, the diversity of communities in habitats that impose the abiotic and biotic stresses described here. What remains to be determined is the genetic and biochemical basis of the symbiotic communication responsible for stress tolerance. Once that is defined, it may be possible to better understand plant community structure and ecosystem dynamics.

In the book Darwin's Blind Spot (Ryan, 2002), Frank Ryan describes how symbiogenesis is a diversion from Darwinian evolution. Here, we demonstrate that habitat-adapted symbiosis may be a common non-Darwinian phenomenon in plant biology and is an important component of plant adaptation to environmental stresses. In the light of these results, fundamental questions in plant biology need to be addressed: (1) Can plants adapt to high-stress habitats in the absence of fungal endophytes? (2) Do fungal endophytes regulate plant community structure by selectivity at the level of host colonization or communication? (3) What are the genetic and biochemical basis of symbiotic communication responsible for stress tolerance? These questions will be addressed in future studies to further elucidate the role of class 2 endophytes in the evolution, ecology, biodiversity and distribution patterns of plants.

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