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ARTICLE

Involvement of Arbuscular Mycorrhizal Symbiosis in the Distribution of Sawgrass and Cattail in Florida Everglades

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Abstract In areas of the Florida Everglades, USA, a massive replacement of the historically predominant sawgrass by native cattail is occurring. Phosphorus enrichment due to runoff and hydrological engineering is considered a major environmental cause. As part of our investigation into the genetic and molecular mechanisms underlying this habitat shift, we examined the possible involvement of arbuscular mycorrhizal (AM) symbiosis in sawgrass and cattail. Laboratory experiments determined that sawgrass, but not cattail, was susceptible to fungal inoculation and formed AM under low phosphate (Pi) conditions. Collection of plants from four representative sites in the Everglades revealed that while all sawgrass plants formed root AM associations, no AM was detected in cattail. We identified a phosphate transporter gene of sawgrass, CjPT4, that was preferentially expressed in roots of fungal inoculated and AM plants. In contrast, cattail PT genes were steadily expressed regardless of Pi levels. Our studies demonstrate a strong possibility that ability to form AM symbiosis is a key genetic distinction between sawgrass and cattail in their adaptive response to the changing phosphorus environment. We propose a mechanistic explanation based on AM symbiosis for the distribution and competition of these two plants in the pre-industrial Pi-deficient and modern Pi-enriched Florida Everglades ecosystems.

Keywords *Cladium jamaicense* · Fungi · Gene expression · Phosphate (Pi) · Phosphate transporters · *Typha domingensis*

Introduction

Phosphorus (P) is an essential nutrient for plant growth and development, yet the useful form of phosphorus, inorganic orthophosphate (Pi), is the least available in the soil (Raghothama 1999). Plants have evolved various strategies to enhance Pi acquisition (Raghothama 1999; Rausch and Bucher 2002; Lin et al. 2009). The symbiosis of roots with arbuscular mycorrhizal (AM) fungi is one of the most significant mechanisms. Over 80% of angiosperm plants are believed to form symbiotic relationships with some 150 fungal species of the phylum *Glomeromycota* (Schüβler et al. 2001; Karandashov and Bucher 2005; Bonfante and Genre 2008). Arbuscular mycorrhizal fungi are obligate biotrophs that form long-term feeding (mutualistic or parasitic) relationships with the root cells of their hosts and help plants access mineral nutrients such as Pi; in return the fungi receive carbon (e.g., glucose) from their host plants (Bago et al. 2000; Smith et al. 2001; Javot et al. 2007). At the ecological level, studies have shown significant impacts of AM symbiosis on plant community composition and diversity as well as positive correlations between AM fungal diversity and plant biodiversity, ecosystem variability, and productivity (Grime et al. 1987; Johnson et al. 1997; Smith and Read 1997; van der Heijden et al. 1998).

Since Pi concentration in the soil is much lower (up to 10,000-fold lower) than that in plant cells (Raghothama 1999), Pi uptake requires active transport. Plant roots employ various ATP-driven phosphate transporters to combat this steep concentration gradient and also to carry

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out intra-plant Pi transport (Raghothama 1999; Rausch and Bucher 2002). Among the large number of phosphate transporter genes that plant possess, the so-called high affinity Pi-transporters (PT) are the primary proteins responsible for the uptake of soil Pi by root cells. Environmental factors such as soil Pi levels and AM symbiosis influence PT gene expression. For example, several PT genes in the model plant *Arabidopsis* are specifically induced by Pi-deficient and repressed by Pi-sufficient conditions (Muchhal and Raghothama 1999; Karthikeyan et al. 2002; Mudge et al. 2002). For many mycorrhizal plants (*Arabidopsis* is one exception), AM symbiosis is an important and ubiquitous avenue for Pi acquisition. Although the molecular mechanisms underlying the phosphate translocation between fungi and host plants in AM symbiosis are not yet fully known, the fungi-plant interactions have been well studied. These AM fungi can generate an extensive web of extraradical hyphae and therefore are able to acquire Pi from regions of the soil far beyond root depletion zones. In AM roots, the fungi release Pi from differentiated hyphae (arbuscules) that is impermeable to the plant cytoplasm due to the barrier of the periarbuscular membrane (Smith and Smith 1997; Smith et al. 2001). To obtain this source of Pi, the host plant must be able to transport the phosphate across the periarbuscular membrane to the cortical cells in roots. To do this, mycorrhizal plants have evolved a specific set of phosphate transporters that respond only to AM fungal association. This type of PT proteins has been identified in plants such as *Medicago truncatula* (Harrison et al. 2002), rice (Paszkowski et al. 2002), wheat and maize (Glassop et al. 2005), potato (Rausch et al. 2001; Nagy et al. 2005), and tomato (Xu et al. 2007). They are typically induced by AM fungal infection or found in AM-forming roots and are not responsive to environmental Pi. A study found that the lysolipid lysophosphatidylcholine identified from the root extracts of mycorrhizal potato plants induces the AM-specific PT genes, suggesting lysophospholipids as signals in the AM symbiosis (Drissner et al. 2007). Another study identified lipochitooligosaccharides secreted from the *Glomus* fungi as AM symbiotic signals in Fabaceae, Asteraceae, and Umbelliferae (Maillet et al. 2011). As for the host, a legume gene Vapyrin was found to be essential for the establishment of AM symbiosis and was also required for rhizobial colonization and nodulation (Pumplin et al. 2010; Murray et al. 2011). Whether the lipid- and Vapyrin-mediated AM symbiosis occurs similarly in wetland plants like cattail and sawgrass has not been established.

The Florida Everglades is the largest subtropical freshwater wetland in the United States. Historically, the Everglades was P-limited with levels as low as 10 ppb (~0.3 μM) in water (Davis and Ogden 1994). However, hydrological engineering and urban development over the past century have drastically changed the Everglades ecosystem. P enrichment as a result of runoff from surrounding agricultural and urban areas has instigated the replacement of sawgrass by cattail. Cattail (*Typha domingensis*) and sawgrass (*Cladium jamaicense*) are native emergent plants in the Everglades. In the 1900s, sawgrass marshes covered 60~70% of the area, whereas cattail was only sparsely distributed (Davis and Ogden 1994). Today, massive replacement of sawgrass marshes by cattail in areas of the Everglades has threatened the natural food chain and ecosystem stability (Rutchey and Vilcheck 1999). Many studies have linked P enrichment in soil/water to plant habitat change in the Everglades. For example, a positive correlation was found between cattail abundance and soil P concentration, whereas sawgrass coverage was inversely related to P levels (Newman et al. 1996; Doren et al. 1997; Miao and Sklar 1998; Miao and Debusk 1999; Miao et al. 2000; Brix et al. 2010).

It is known that sawgrass has a low rate of seed germination, slow population growth, low reproductive yield, and a long life cycle (Davis 1991; Miao and Sklar 1998; Webb et al. 2009). On the other hand, cattail is an opportunistic species with high population growth and reproduction rates, long-distance seed dispersal, a short life cycle, and a high biomass turnover rate (Davis 1991; Newman et al. 1996; Miao and DeBusk 1999; Miao 2004). Furthermore, sawgrass and cattail exhibit distinct kinetics for Pi uptake (Newman et al. 1996; Lorenzen et al. 2001; Miao 2004; Brix et al. 2010). The fact that sawgrass occupies Pi-deficient areas while cattail replaces sawgrass in Pi-enriched area suggests that these plants have contrasting life histories with regard to Pi. One major difference between sawgrass and cattail may be their propensity to form symbiotic AM relationships.

Although AM symbiosis has been studied in many plants (see review by Bago et al. 2000), little is known about AM-related gene activity in wetland ecosystems. In fact, whether plants in the Everglades even form AM symbiosis is a matter of dispute. Meador (1977) concluded that the Everglades is a unique non-mycorrhizal ecosystem. However, more recent assessments have not only found AM fungi in Everglades-grown sawgrass roots (Aziz et al. 1995), but also demonstrated that AM fungi can increase sawgrass growth and Pi uptake in greenhouse experiments (Jayachandran and Shetty 2003). As for cattail, greenhouse studies have shown that all three cattail species in North America, *Typha latifolia*, *T. angustifolia*, and *T. domingensis*, can be infected by AM fungi and form AM (Stenlund and Charvat 1994; Tang et al. 2001; Ray and Inouye 2006). However, Cornwell et al. (2001) found *T. latifolia* to be non-mycorrhizal in P-deficient wetland sites. Thus our knowledge is acutely lacking regarding AM symbiosis in emergent/aquatic plants such as sawgrass and cattail in wetland ecosystems. While a Pi-deficient environment is seemingly

a prerequisite for successful colonization of AM fungi, host plants must also possess the necessary genetic makeup involved in AM formation. Furthermore, because AM fungi are obligate aerobes, the long hydroperiod of wetland ecosystems suppresses fungal colonization in roots. The fact that sawgrass is adapted to a Pi-deficient environment with a low water table and cattail often grows in Pi-sufficient, water saturated areas (Davis 1991; Miao and Sklar 1998), indicates that AM symbiosis likely played a more significant evolutionary role in the adaptation of sawgrass than cattail to the pre-industrial, Pi-deficient Everglades. Almost paradoxically, the two most influential factors (Pi and hydrology) for AM formation have been significantly altered by recent human activity. The combinatorial effect of these factors may have inadvertently changed downstream AM-plant symbiosis, Pi uptake, and spatial distribution of cattail and sawgrass in the Everglades. In an effort to address this question, we report our investigations of AM symbiosis in sawgrass and cattail from the Everglades and its implication in phosphate uptake.

Methods

Seed Germination and Plant Growth in Lab

Seeds of sawgrass (*Cladium jamaicense*) and cattail (*Typha domingensis*) were collected from the Water Conservation Areas (WCA) 2A, west of Boca Raton, Florida, USA, in the Florida Everglades (Fig. 1) and stored at 4°C until use. Sawgrass seeds were surface sterilized for three days in 10% bleach solution and rinsed with distilled water three times (Webb et al. 2009). Cattail seeds were sterilized in 10% bleach solution for 20 min and rinsed with distilled water three times. Seeds were germinated in solid Hoagland medium and placed in a growth chamber (AR-36, Percival) under a 16-hr light, 8-hr dark photoperiod and a diurnal temperature cycle of 25°C light-15°C dark. Daytime light intensity was approximately 250 μmole/m^2/s. The 40-day-old plants were transferred to pots containing autoclaved soil and grown in a growth chamber under a 16-hr light, 8-hr dark photoperiod, 24°C, and cool white light with an intensity of approximately 250 μmole/m^2/s. Each pot was watered weekly with 100 mL half-strength Hoagland solution (pH 6.5; Online Table S1) having Pi (orthophosphate) concentrations of either 5 μM (designated low-Pi) or 1000 μM (designated high-Pi).

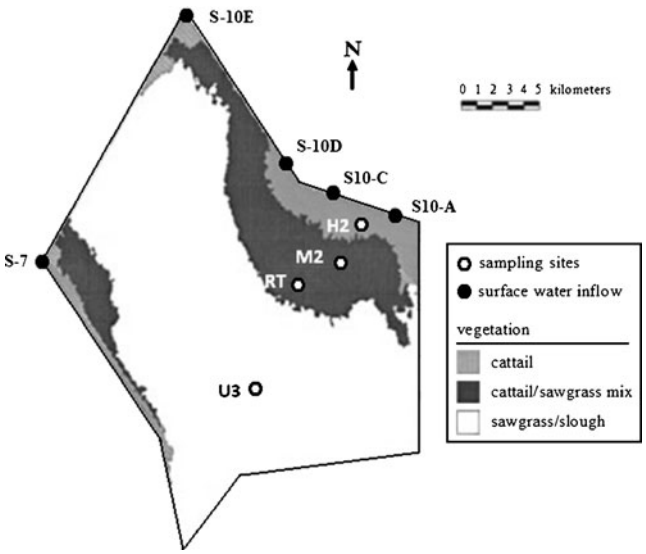

Fig. 1 Sites (H2, M2, RT and U3) for collecting field samples in WCA-2A of the Florida Everglades. The map was adapted from Rutchey and Vilcheck (1999) and Thomas et al. (2009)

Arbuscular Mycorrhizal (AM) Fungal Inoculation

Once the plants were established, fungi of the genus *Glomus* were inoculated. *Glomus* were obtained from Fungi Perfecti, LLC and contained fine spores of *Glomus aggregatum, G. etunicatum, G. intraradices*, and *G. mosseae*. These species are known to form symbiotic AM relationships with many plants (Schüβler et al. 2001). Fungi were mixed into water at a concentration of 6 mL spores per liter of water. The phosphate concentration of the fungal solution was assayed and determined to be negligible. A single 200 mL inoculation of fungal solution was applied onto the shoot/root junction area of each pot. Control plants were treated with 200 mL water. Both AM and control plants were then maintained under the Pi treatment regimes described previously. Treatments were performed in triplicate. Roots were examined for mycorrhizal colonization one month after inoculation. Previous laboratory studies have shown that AM colonization occurs as little as two weeks, but optimally 4 weeks, after inoculation (Harrison et al. 2002; Paszkowski et al. 2002).

Field Sample Collection

Four representative research sites established and maintained by South Florida Water Management District researchers were selected for cattail and sawgrass sample collection (Fig. 1). Site H2 represented an impacted area with high P concentration (eutrophic; cattail dominated); site RT represented a transitional area with medium P (cattail-sawgrass mix); site U3 represented a non-impacted, reference area with low Pi (oligotrophic; sawgrass dominated); and site M2 represents a dry area with medium P (Thomas et al. 2009). The soil P levels in those sites are presented in Table 1. At least three whole plants separated by at least 10 m were taken at each site for each species.

Table 1 Percentage of AM colonization in sawgrass and cattail roots

Sample locale	P and water conditions	% colonization (no. of samples examined)		
		Sawgrass		Cattail
		Primary roots	Secondary roots	Roots
lab	low Pi (5 μM), no visible water	13.0 (n=23)	14.7 (n=34)	0 (n=48)
lab	high Pi (1000 μM), no visible water	0 (n=18)	0 (n=32)	0 (n=32)
site U3[a]	low P (600 mg P/kg soil), submerge	21.7 (n=23)	33.3 (n=75)	0 (n=84)
site RT[a]	medium P (600–1000 mg P/kg soil), submerge	20.0 (n=20)	19.1 (n=84)	0 (n=65)
site H2[a]	high P (1000–1200 mg P/kg soil), submerge	16.1 (n=31)	17.4 (n=46)	0 (n=92)
site M2[a]	medium P (600–1000 mg P/kg soil), no visible water	18.5 (n=27)	19.3 (n=119)	0 (n=85)

[a] Total P levels in the Everglades sites (Fig. 1) are based on characterizations by Thomas et al. (2009).

Microscopic Examination of Mycorrhizal Colonization

Segments (2–3 cm in length) of different root sections were sliced, washed thoroughly with water and boiled in 10% (w/v) KOH for 10 to 15 min. Cleared roots were washed with water several times and placed in 1% HCl for 5 min. Several staining methods (Philips and Hayman 1970; Vierheilig et al. 2005; Tang et al. 2001; Jayachandran and Shetty 2003; Ray and Inouye 2006) were tested. After HCl was removed, the roots were boiled in 0.05% trypan blue (MP Biomedicals, LLC) for 10 min, 0.05% acid fuchsin (Electron Microscopy Sciences) for 25 min, or 0.05% CBE (Acros Organics) for 20 min. All the dyes were dissolved in lactoglycerol (lactic acid: glycerol: water, 1:1:1, v/v). Roots were then destained in 50% glycerol or water. For the ink method, cleared roots were first stained in a 5% blue ink (Bristol, Part No. 19084)-acetic acid solution by boiling for 20 min and then destained in water with a few drops of acetic acid. Roots were mounted on slides and inspected with a digital camera linked stereomicroscope (1274ZH, VanGuard). Percent colonization was quantified as the number of AM fungal structures per plate divided by the total number of roots (Stenlund and Charvat 1994).

Plant Growth Assay and Phosphate Determination

Shoots and roots of AM-inoculated and control plants were separated and the fresh weight of each portion was recorded. Then plants were dried at 90°C for five days for dry mass measurement. Dried tissue was ground en masse and representative aliquots were heated at 500°C for six hours. Ashed tissue was dissolved in 0.5 N HCl and phosphorus assayed using the procedures of Ames (1966). Mean total weight and mean total cellular phosphate were compared among treatments using one-way ANOVAs with Tukey's post-hoc. Significance was set at α=0.05.

DNA and RNA Isolation, cDNA Synthesis, and Cloning

Total cellular DNA was isolated from shoots of sawgrass and cattail using the DNeasy plant kit (Qiagen). Total RNA was isolated from roots and shoots of sawgrass and cattail using the RNeasy plant kit (Qiagen). On-column digestion with RNase-free DNase was done to remove residual DNA. First strand cDNA was synthesized from up to 2 μg of total RNA by using a cDNA synthesis kit (Fermentas) and oligo $(dT)_{17}$-AP, according to the manufacturer's instruction, resulting in generation of cDNA libraries. The AP adaptor facilitated downstream cloning of 3′-region of the transcripts. PCR for the actin gene (ACT) was used to monitor the quality and quantity of the cDNA libraries.

To clone phosphate transporter (PT) genes, universal primers (Online Table S2) were designed based on the highly conserved segments inferred from sequences from other plants available in the public databases. Polymerase chain reactions (PCR) were performed using the genomic DNA or cDNA from sawgrass and cattail as the template. The PCR-generated DNA fragments were cloned into plasmid vector pCR®-Blunt (Invitrogen) and sequenced (Genomics Core Facility, Northwestern University). Based on the newly acquired sequences, species-specific primers were designed. PCR walking (Cottage et al. 2001; Liu and Chen 2007) and/or PCR using oligo dT-adaptor primers were employed to clone 5′- and 3′-regions that were then sequenced. Sequence analysis was done using public databases and Biology WorkBench (San Diego Supercomputer Center).

Semi-quantitative Reverse Transcription (RT)-PCR

The cDNA libraries were generated using total RNA as described above. For sawgrass, gene-specific primers (Online Table S3) were used to study PT gene expression with an equal aliquot from a single master reaction solution

containing all the reactants except for primers. This way, the relative transcript level of each PT gene was assessed for individual treatment/plant. For cattail, PT transcript levels were titrated by increasing the PCR cycle number. The housekeeping genes RPS16 (a 40S ribosomal protein) and ACT, and the Pi-responsive acid phosphatase gene (ACP) from cattail were used as controls.

Results

Sawgrass, but not Cattail, Easily Forms AM Symbiosis

Of the four commonly used staining methods for AM detection tested, comparable staining results were given by trypan blue, acid fuchsin, and ink, but not CBE. Trypan blue images presented the highest quality and were used to determine percent colonization one month after inoculation (Table 1). Representative images are provided in Fig. 2. Darkly stained areas, most apparent in the intercellular space, suggested the presence of AM fungal hyphae and colonization, whereas the non-AM forming tissues gave a clear, even background, consistent with other published studies. Interestingly, *in situ* presence of AM fungal symbiosis was observed in the roots of low-Pi grown sawgrass, but not in the roots of high-Pi grown sawgrass, nor cattail roots regardless of Pi treatments. No significant difference was found in the percent colonization of primary and secondary roots of low-Pi grown sawgrass (Table 1). Root examination three months after inoculation showed similar results.

Consistent with our laboratory observations, in field samples collected from experimental sites in the Everglades, the apparent presence of AM was detected in all samples of sawgrass but not in cattail (Fig. 2 and Online Fig. S1; Table 1). There appears a general negative correlation between soil P level and the occurrence of AM colonization. Particularly for the secondary roots the AM colonization was significantly different between the low Pi site U3 and other sites (Table 1). Water levels did not seem to be a major factor for colonization, as shown by comparison between sites RT and M2 (Table 1).

AM Symbiosis Enhances Growth but not Phosphate Accumulation in Sawgrass

Under laboratory conditions, cattail growth in soil was significantly higher than sawgrass and not influenced by Pi treatment (Fig. 3a). While this might indicate soil Pi concentration was already overly sufficient, the validity of our treatments was apparent in the correlation between total cellular P content and Pi treatment observed in cattail (Fig. 3b). At the level of species and organ type, significant differences in growth (Fig. 3a) and phenotype (Online Fig. S2) were only apparent in sawgrass shoots. Interestingly, the increased shoot growth in sawgrass appeared to be a result of inoculation with AM fungi. The phenotypic observation that sawgrass possessing AM had longer leaves than both un-inoculated plants and AM-lacking plants provided further support for the benefits of symbiosis (Online Fig. S3). Surprisingly, under high Pi condition, inoculated sawgrass plants without noticeable AM formation (Fig. 2) had a higher biomass than

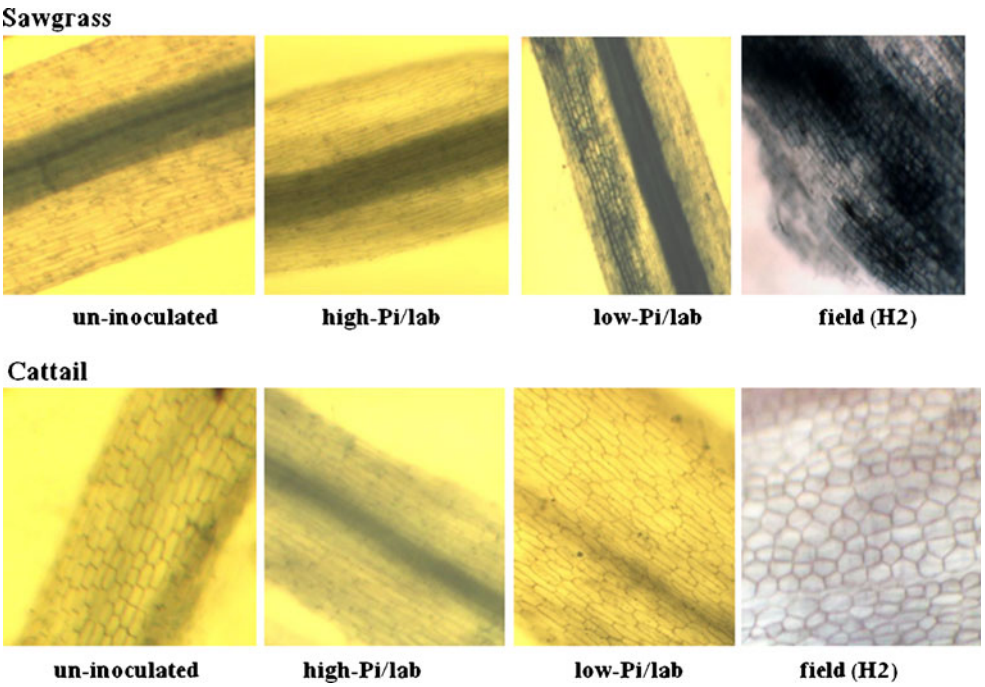

Fig. 2 Representative photomicrographs of staining for arbuscular mycorrhiza (AM) in roots of sawgrass and cattail. AM was detected in sawgrass roots of both low Pi-grown lab samples and field samples from all four sites (Site H2 is shown. Other sites are shown in Online Fig. S1). No AM was detected in cattail roots

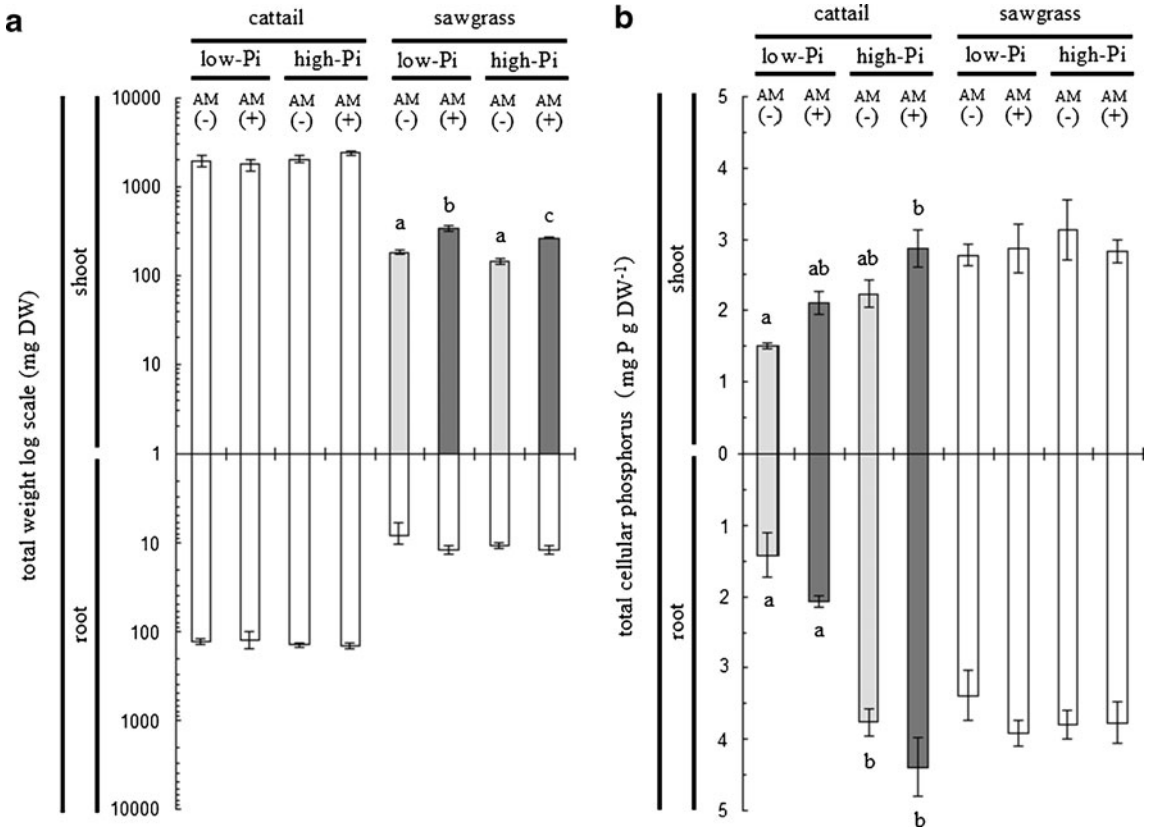

Fig. 3 Effect of AM fungal inoculation on biomass (**a**) and total cellular phosphate (**b**) of cattail and sawgrass. Although fungal inoculation had no significant effect on growth of cattail, AM formation significantly increased sawgrass growth independent of Pi availability. A correlation between total P with increasing Pi availability was observed in cattail, but not in sawgrass. Significance (α=0.05) was assessed at the level of phosphate availability and AM inoculation. Significantly different values are highlighted and annotated. Values are the means ± standard error of three replicates (n=3)

un-inoculated plants (Fig. 3a). In the light of these observations, it was noteworthy that although cattail accumulated more P under high-Pi, sawgrass showed no significant changes in P accumulation regardless of AM inoculation or Pi level (Fig. 3b).

Although not statistically significant, the shoots and roots of cattail appeared to accumulate more P when inoculated with AM fungi, even without AM colonization (Fig. 3b). Although this observation may simply reflect the range of variation for sampling and measurement, it is also possible that the fungal inoculum may temporarily increase Pi availability by remobilizing the otherwise insoluble P in soil (Smith and Read 1997).

AM Symbiosis Activates the Expression of a Phosphate Transporter Gene in Sawgrass

Phosphate transporters are directly involved in Pi uptake by roots. Like other plants, sawgrass and cattail possess multiple PT genes (Online Fig. S4). We cloned and sequenced several PT gene products from the AM fungal-infected sawgrass. Based on the sequence variation in the coding and 3′-untranslated regions, we were able to identify seven distinct members of the PT gene family in sawgrass (Fig. 4a). Four different mRNA species identified from roots were named CjPT1~4. The other three sequences isolated from genomic DNA or shoot cDNA were designated CjPT5~7. Phylogenetic analysis revealed that CjPT4 was clustered with the rice AM-specific OsPT11 but not the Pi-responsive OsPT2 (Fig. 4b), inferring a possible AM regulation of the CjPT4 gene expression.

Using specific primers, we studied the relative expression level of these four PT genes (CjPT1~4) to examine whether any of them responded to AM and Pi treatments. Using an equal aliquot from a single master reaction and the same amplification parameters, we were able to reliably assess the relative transcript level of each PT gene in individual treatment/plant (but not between two plants). Our results showed that CjPT1, CjPT2, and CjPT3 genes were similarly expressed in both shoots and roots at a relatively high level regardless of sample origin, fungal treatment, or Pi level (Fig. 5). More importantly, the transcript level of CjPT4 was increased in AM-forming roots of both lab-grown and field-collected plants, whereas

Fig. 4 **a** Sequence comparison and **b** phylogenetic relationship of sawgrass phosphate transporters (CjPT). The more informative C-terminal portions of the sequences were analyzed. One-letter symbols for amino acids are used. * identical residues; : highly similar residues; . weakly similar residues; - gaps to maximize the match. The rice Pi-responsive OsPT2 and AM-specific OsPT11 (Paszkowski et al. 2002) are used as reference

A.

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CjPT1    MMAVFMLGLAIPYHHWTTPGNHIGFAVMYAFTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAAA
CjPT2    MMTVFMLGLAIPYHHWTTKGNHIGFVVMYALTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAAS
CjPT3    MMTVFMLGLAVPYHHWTIAGNHTGFVVMYGLTFFFANFGPNSTTFIVPAEVFPARLRSTCHGISAAA
CjPT4    MMTVFMLGLAVPYHHWTTAGHQIGFVVMYAFTFFFANFGPNATTFIVPAEIFPARLRSTCHGISAAS
CjPT5    MMTVFMLGLAIPYHHWTTAGNHIGFIVMYGFTFFFANFGPNSTTFIVPAEIFPARLRSTCHGISAAS
CjPT6    MMTVFMLGPAIPHHHRTTPGNHISLVVLYGLTFFFANFGPNSTTFIVPAEIFPARPRSTCHGISAAA
CjPT7    MMTVFMLGLAVPYHHRTTPGQHTGFVVMYGLTFFFANFGPNATTFIVPAEIFPARLRSTCHGISAAR
         **:***** *:*:** *  *:: .: *:*.:**********:********:**** **********

CjPT1    GKAGAIIGSFGFLYAAQDQDKTKTDHGYPPGIGVRNSLFVLAGCNFLGLVFTFLAPESKGKSLEELS
CjPT2    GKAGAIVGAFGFLYAAQSTDPAKTDPGYPTGIGIRNSLFLLAGCNLIGVFFTFFVPDANGKSLEEAS
CjPT3    GKAGAIIGAFGFLYASQGKTPETRDRGYPKGIGLRNSLFVLAVSNFLGMVMSLFVPEAMGKSLEEIS
CjPT4    GKAGAIVGAFGFLYASQGKAPDSRDAGYPAGIGVKNSLFVLAGCNLLGFFFTFLVPEPKGKSLEEIS
CjPT5    GKLGAIIGSFGFLYLAQNPDPAKADHGYPPGIGVRNSLFLLAGCNFLGLILTFLVPESKGKSLEEIS
CjPT6    GKAGAITGSFGFLYAAQNQNKALADHGYPAGIGVRNSLFVLATCNLLGLIFTCLVPESNGKSLEELS
CjPT7    GKAGAVVGAFGFLYASQGRTP---DRGYPKGIGLRNSLFVLAASNFLGMVMTIFVPEAKGKSLKEMT
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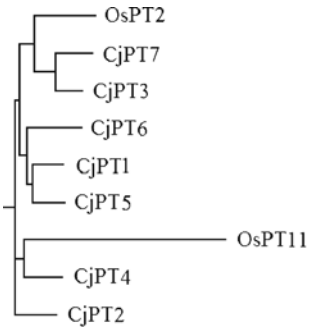
B.



non-AM roots showed a lower level of expression (Fig. 5). Although not conclusive, these results suggest up-regulation of CjPT4 gene by AM symbiosis and its role in fungi-associated Pi uptake in sawgrass.

Since AM infection was not found in both lab-grown and Everglades-collected cattail plants, as expected an AM fungi-regulated PT gene was not found in cattail, using similar experimental approaches as for sawgrass. As shown in Fig. 6, the expression of cattail PT genes was not affected by Pi levels, a pattern similar to the housekeeping genes RPS16 and ACT. By contrast, the cattail acid

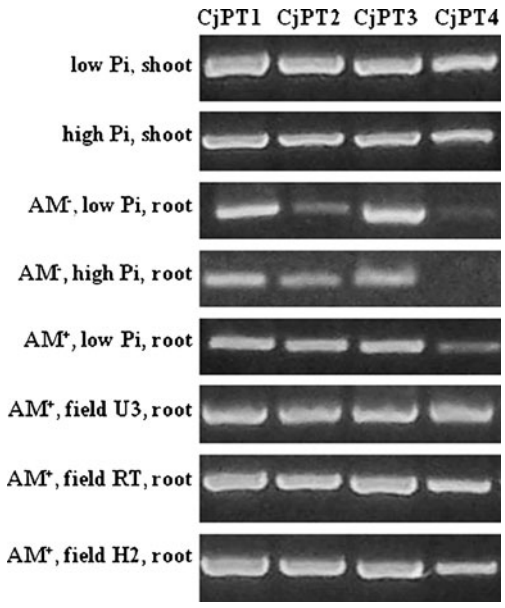


Fig. 5 Relative expression of four phosphate transporter genes (CjPT1~4) in sawgrass. Each panel represents four individual semi-quantitative RT-PCR reactions from a single master mixture containing all reactants except for gene-specific primers. CjPT4 is likely AM-induced in roots

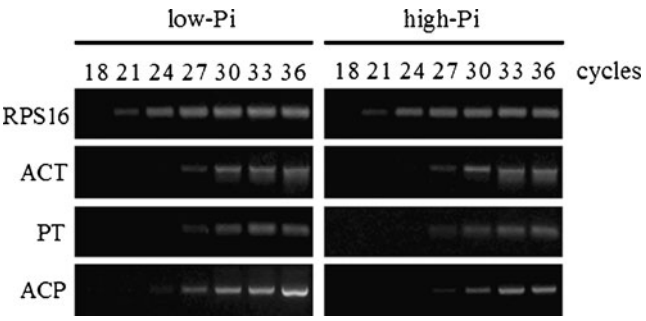


Fig. 6 Phosphate transporter (PT) gene expression in roots of laboratory-cultured cattail plants. The gene expression of PT, RPS16 (a 40S ribosomal protein) and ACT (actin) was not influenced by Pi, but acid phosphatase gene (ACP) was activated in response to low-Pi

phosphatase gene (ACP), which is also involved in P uptake, was up-regulated by low-Pi (Fig. 6), demonstrating that different genes respond to Pi treatments differently.

Discussion

Even though our photomicrographs did not show more clearly defined hyphal structures in the AM forming samples, the clear contrast between AM positive and AM negative samples demonstrated the validity of the staining methods. In our laboratory study, we found that fungal inoculation could induce the formation of AM structure in sawgrass roots at low-Pi but not at high-Pi. We did not find AM in cattail at either Pi levels. AM fungal symbiosis significantly increased sawgrass shoot biomass, which is consistent with the results of Jayachandran and Shetty (2003). In our examination of field samples, we detected AM symbiosis in all sawgrass plants but not in cattail. Our observations appear to suggest a negative correlation between soil P level and the occurrence of AM colonization, particularly for secondary roots (Table 1). We did not detect a significant impact of water levels on AM colonization. Ray and Inouye (2006) reported AM colonization in *T. latifolia* during both flooding and dry periods. Similarly, Miller and Sharitz (2000) found that even though flooding reduced the initiation of colonization in two semiaquatic grasses, *Panicum hemitomon* and *Leersia hexandra*, it had minimal effects once AM colonization was established. Although our field study agrees with those reports, continued study over an extended period is needed for a more definitive conclusion for our field sites.

Collectively, our lab and field results indicate (1) sawgrass is highly susceptible to AM fungal infection and easily forms AM, and (2) high Pi hinders AM colonization, indicating that AM fungi could play an active role in nutrient acquisition for sawgrass when Pi is deficient. The Everglades has been historically Pi scarce. Even those so-called Pi-enriched areas (water Pi concentrations as high as 40 μM; Koch and Reddy 1992) have a much lower sustaining Pi level than the AM-suppressing condition we used in the lab. This explains the occurrence of AM symbiosis in all samples examined where field Pi levels may not have been high enough to inhibit AM formation. Our results support the notion that sawgrass plants in the Florida Everglades employ AM symbiosis as a means of uptake of mineral nutrients such as Pi.

Phosphate transporters are directly involved in Pi uptake and mobilization (Raghothama 1999). In support for AM fungal symbiosis in sawgrass, we identified a root phosphate transporter gene CjPT4 preferentially up-regulated by AM fungal infection. However, unlike the AM-specific PT genes that are exclusively expressed in mycorrhizal roots of *Medicago truncatula*, rice, tomato, and potato (Harrison et al. 2002; Paszkowski et al. 2002; Nagy et al. 2005), CjPT4 was also expressed in shoots and at a low level in non-mycorrhizal roots. It is more similar to the barley and maize AM-specific PT that is also expressed in other tissues/organs (Glassop et al. 2005). No matter its spatial expression pattern, CjPT4 likely contributes to AM- associated P uptake in sawgrass roots. The biochemical and physiological activities of CjPT4 remain to be studied further. Nonetheless, the apparent involvement of fungi in modulating PT gene expression in the host sawgrass provides a molecular mechanism/explanation for its adaptation to the Pi-deficient wetland ecosystem.

In stark contrast to sawgrass, our studies did not find mycorrhizae in either lab-grown or field-collected cattail plants, even when they shared the wetland habitat with sawgrass and were collected from the same sites. Our data highlight a strong possibility that cattail (*T. domingensis*) in the Everglades ecosystem may not employ AM symbiosis for phosphate acquisition. Previously, Meador (1977) could not find any mycorrhizal plants in the Florida Everglades. More recently, Cornwell et al. (2001) also found the cattail *T. latifolia* to be non-mycorrhizal in an AM-favorable P-deficient wetland site. Yet other studies of field-collected and fungal inoculated samples found various levels of AM colonization in *T. latifolia*, *T. angustifolia*, and hybrid cattail (Stenlund and Charvat 1994; Tang et al. 2001; Ray and Inouye 2006). It is likely that establishment of AM symbiosis in cattail plants depends on various factors such as geological locations, seasons, nutrient and water conditions, and fungal and cattail species. The absence of AM in our lab-treated cattail samples might be attributed in part to factors such as fungal species specificity or inoculation duration. Nonetheless, the lack of AM in the field samples provided strong indication of cattail's inability to form AM symbiosis, thus one less avenue with which to acquire phosphorus. In the context of the current large-scale invasion of cattail into Pi-enriched areas of the Everglades, our findings offer a new genetic/molecular explanation of the habitat shift. Since resource availability is often a driver of mycorrhizal symbiosis in local plant adaptation (Johnson et al. 2010), AM association may reflect aspects of genetic differences and history of environmental changes that sawgrass and cattail have encountered in the Everglades.

Sawgrass and cattail have different life histories, growth and reproduction strategies, and non-mycorrhizal Pi uptake kinetics (Davis 1991; Newman et al. 1996; Miao and DeBusk 1999; Miao et al. 2000; Miao 2004; Webb et al. 2009; Brix et al. 2010). We propose a scenario of sawgrass-cattail relationship: under low Pi condition in the pre-1900s Everglades and the current so-called non-impacted areas in the Everglades, sawgrass employs an array of mechanisms for efficient uptake, utilization, and conservation of

phosphorus. With regard to phosphate transporters (PT), most PT genes in both sawgrass and cattail are largely unresponsive to changes in the environmental P levels. However, up-regulation of the AM-responsive PT gene accompanying AM symbiosis in sawgrass would provide an advantage over the mycorrhizal deficient cattail in Pi impoverished soils. The carbon cost of AM symbiosis—in a form of 4~20% of photosynthetically sequestered carbon the host plant must provide to the fungi (Bago et al. 2000)—is likely offset in P-impoverished environments thereby generating an overall benefit to species (such as sawgrass) employing such strategies. In conjunction with AM symbiosis, sawgrass is also less responsive to changes in resource availability and maintains a steady growth pattern almost independent of Pi availability (Miao et al. 1997; Miao and Sklar 1998; this study). All these characters contribute to sawgrass' adaptive ability to survive in the P-deficient environment and may explain its more-or-less monoculture predominance in the pre-industrial Everglades. In contrast, cattail is plastic in biomass and nutrient allocation under varying Pi levels and responds to low Pi levels by allocating more biomass to roots, developing more advanced secondary root systems and slowing shoot growth (Miao and Sklar 1998; Miao et al. 2000; Miao 2004; Brix et al. 2010) and maintaining a steady state of PT activity (this study). Therefore, cattail plants are able to maintain a level of Pi uptake to sustain growth and reproduction, which may explain its presence, however spotty, along with the predominant sawgrass in the pre-1900s extremely Pi-deficient Everglades. Lack of AM fungal symbiosis likely contributed to its sparse distribution in the Pi-deficient Everglades.

However, with anthropogenic Pi-enrichment, the increasing ease with which plants can acquire their "own" phosphate negates the apparent "lack-of-AM" disadvantage in cattail. The competitive pressure shifts to the P-uptake capacities of plants and favors those capable of rapid Pi-uptake like cattail. Cattail PT genes are expressed steadily regardless of Pi levels, an indication of an active Pi uptake machinery that lacks Pi feedback regulation. Thus, cattail can make full use of the newly available resources for rapid shoot growth (Lorenzen et al. 2001), conceivably increasing photosynthetic activity. Its high growth rates, short life cycles, high seed production, long-distance seed distribution, and high biomass turnover rates all facilitate the invasion and establishment in the Pi-enriched areas in the Everglades. On the other hand, AM symbiosis in sawgrass no longer generates appreciative benefit when Pi is plentiful. Compounded by perpetual low seed production and germination (Webb et al. 2009), sawgrass would eventually lose ground to the encroaching cattail. It is likely that restricting Pi availability will significantly help to retard the invasion by opportunistic cattail, to favor the growth of the AM-empowered sawgrass, and to restore the historic pattern of co-habitation by sawgrass and cattail in the Florida Everglades.

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