Journal of Plant Physiology 167 (2010) 1009–1017

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Piriformospora indica confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein

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ARTICLE INFO

Article history:
Received 9 December 2009
Received in revised form 3 February 2010
Accepted 3 February 2010

Keywords:
Chinese cabbage
Drought tolerance
Antioxidant enzymes
Ca^{2+}-sensing regulator
Piriformospora indica

ABSTRACT

Piriformospora indica, a root-colonizing endophytic fungus of Sebacinales, promotes plant growth and confers resistance against biotic and abiotic stress. The fungus strongly colonizes the roots of Chinese cabbage, promotes root and shoot growth, and promotes lateral root formation. When colonized plants were exposed to polyethylene glycol to mimic drought stress, the activities of peroxidases, catalases and superoxide dismutases in the leaves were upregulated within 24 h. The fungus retarded the drought-induced decline in the photosynthetic efficiency and the degradation of chlorophylls and thylakoid proteins. The expression levels of the drought-related genes *DREB2A*, *CBL1*, *ANAC072* and *RD29A* were upregulated in the drought-stressed leaves of colonized plants. Furthermore, the CAS mRNA level for the thylakoid membrane associated Ca^{2+}-sensing regulator and the amount of the CAS protein increased. We conclude that antioxidant enzyme activities, drought-related genes and CAS are three crucial targets of *P. indica* in Chinese cabbage leaves during the establishment of drought tolerance. *P. indica*-colonized Chinese cabbage provides a good model system to study root-to-shoot communication.

1. Introduction

The endophytic fungus *Piriformospora indica* colonizes the roots of many plant species, promotes their growth and seed yield and confers tolerance against biotic and abiotic stress (Varma et al., 1999; Verma et al., 1998; Oelmüller et al., 2009). Although the endophytic interaction of *P. indica* with various plant species shares similarities with mycorrhiza (Harrison, 2005; Oldroyd et al., 2005) and the symbiosis between plants and plant-growth promoting rhizobacteria (Stein et al., 2008; Lugtenberg and Kamilova, 2009), relatively little is known about the molecular mechanisms by which the fungus promotes plant performance. Most studies have been performed with model plants such as *Arabidopsis thaliana* (Peškan-Berghöfer et al., 2004; Pham et al., 2004a,b; Oelmüller et al., 2004, 2009; Shahollari et al., 2005, 2007; Sherameti et al., 2005, 2008a,b; Vadassery et al., 2009a,b), barley (Waller et al., 2005, 2008; Baltruschat et al., 2008) or tobacco (Barazani et al., 2005, 2007, for *Sebacina vermifera*). We found that Chinese cabbage (*Brassica campestris* L. ssp. Chinensis) is a good host for *P. indica*, and that the performance of adult plants is strongly promoted by the fungus, particularly under stress conditions. Chinese cabbage is a fast-growing plant and an important vegetable in China, Japan and Korea. Efficient transformation systems have been established (cf. Vanjildorj et al., 2009). Although the plant is closely related to *Arabidopsis*, the genetic programs controlling the development and habitus of Chinese cabbage must be quite different from those of *Arabidopsis*. Therefore, it is of interest to investigate whether the growth response induced by *P. indica* is mediated by the same signaling events in both species. Furthermore, the fungus confers resistance against drought and leaf pathogens (Johnson et al., manuscript in preparation), suggesting efficient information flow from the colonized roots to the leaves. Here, we report that drought tolerance is associated with the activation of antioxidant enzyme activities, the upregulation of mRNA levels for drought-related proteins, and rapid accumulation of the plastid-localized Ca^{2+}-sensing regulator (CAS) protein in the leaves. CAS might function as a regulator of cytoplasmic Ca^{2+} levels in controlling stomata aperture (Nomura et al., 2008). We identified three targets of the fungus that participate in the establishment of *P. indica*-mediated drought tolerance in Chinese cabbage leaves.

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doi:10.1016/j.jplph.2010.02.013

2. Materials and methods

2.1. Growth and cultivation conditions

P. indica was cultured in Petri dishes on a modified Kaefer medium (Sherameti et al., 2005). The plates were placed in a temperature- and light-controlled growth chamber at 25 °C and 80 μmol s^{-1} m^{-2} with a 12 h photoperiod for 2 weeks. The liquid culture was kept at 50 rpm for 15 d at room temperature in the dark.

Chinese cabbage seeds were surface-sterilized and the seedlings were first kept in glass jars containing 1/2 concentration of MS nutrient medium under sterile conditions (Murashige and Skoog, 1962), 25 °C and a 12 h photoperiod. After 5 days, one plug of *P. indica* mycelium was added to infect the roots of the seedlings. The control seedlings were mock-treated (solid medium without *P. indica*). After 15 days of co-cultivation, the seedlings were transferred to soil for 30 days (1 seedling/pot) before further physiological analyses were performed.

2.2. Analysis of root colonization

15 and 30 days after co-cultivation on soil, root colonization was analyzed under the microscope. The roots were washed thoroughly in running tap water to remove the medium and attached mycelium, cut into 1 cm pieces and treated overnight with 10% KOH solution at room temperature. The roots were washed 5 times with distilled water and treated 4 times with 1% HCl before staining with 0.05% trypan blue. The stained root segments were examined microscopically according to Rai et al. (2001).

To compare the colonization of Chinese cabbage roots with that of, *Arabidopsis* the *Pitef1*/*actin* cDNA ratios were determined as described in Sherameti et al. (2008b). RNA was isolated from colonized roots of the two plant species and, after reverse transcription, the following primer pairs were used to amplify the cDNA fragments: *Pitef1*, gagctgactacggttgttgag and ggagacaatgtcaaggtcgg; *Bcactin1* (*Brassica campestris* Pekinensis, FJ969844) and *Atactin2* (*Arabidopsis thaliana*, At3g01015) ggccgaggctgatgacattcaacc and gcctcggtaagaagaaccgggtgc.

2.3. Determination of biomass parameters

Fifteen or 30 days after co-cultivation with *P. indica* in soil, plants, which were either co-cultivated with *P. indica* or mock-treated, were used to assess various biomass parameters including fresh root and shoot weight and root and shoot lengths. The roots were washed with distilled water. The lengths and the weights of the aerial parts and of the roots were analyzed separately. For the determination of the dry weights, the material was dried overnight in an oven at 80 °C.

2.4. Drought-stress experiments

One month after transfer to soil, *P. indica* and mock-treated Chinese cabbage plants were treated with a 20% polyethyleneglycol 4000 (PEG) solution. Water was used for the control plants. The development of PEG-treated and water-control plants either grown in the absence or presence of the fungus was monitored over the next 96 h. The experiments were repeated 4 times.

2.5. Enzyme assays

Leaf material without the midrib (0.3 g) was homogenized in 4 ml 50 mM PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.8) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA) and 1% polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 24,000 × *g* for 20 min and the supernatant was used for the enzyme assays. Superoxide dismutase (SOD) activity was assayed according to Giannopolitis and Ries (1977) by measuring the reduction of nitrotetrazolium blue chloride at 560 nm. The catalase (CAT) activity was determined spectrometrically as described by Gao (2006) by measuring H2O2 consumption at 240 nm, and the peroxidase (POD) activity was determined as described by Cakmak and Marschner (1992). Oxidation of guaiacol in the presence of H_2O_2 was measured by an increase in the absorbance at 470 nm.

2.6. Determination of the malondialdehyde content

The malondialdehyde content was determined according to Zhang and Fan (2007). The component from the supernatant of the extract was precipitated with 0.5% thiobarbituric acid, the suspension was boiled for 10 min and immediately cooled down on ice. After centrifugation at 8000 × *g* for 10 min, the absorbance was measured at 532 and 600 nm.

2.7. Western analysis

Four days after treatment of the *P. indica*-colonized plants with 20% PEG solution, the leaf material was harvested and ground in liquid nitrogen. Proteins were extracted with 50 mM TRIS pH 7.8, 10 mM $MgCl_2$, 5% SDS. The mixture was incubated at 78 °C for 10 min. After centrifugation, the supernatant was used for protein determination according to Lowry et al. (1951). An equal amount of protein (40 μg) was separated on a one-dimensional SDS gel (Laemmli, 1970). After transfer to PVDF membranes (Amersham Pharmacia Biotech), the membranes were incubated with the respective primary antisera, followed by incubation with a goat anti-rabbit secondary antiserum coupled to peroxidase (Sigma–Aldrich Chemie GmbH). Proteins were visualized by enhanced chemiluminescence. The antisera have been described previously: antibodies against D1, the 33 kDa protein of photosystem II, PsaA, PsaD, PetC, nitrate reductase and ferredoxin in Hein et al. (2009), against PsaF and Hcf101 in Stöckel and Oelmüller (2004) and Stöckel et al. (2006), against the 23 kDa protein of photosystem II in Palomares et al. (1993) and against the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase in Oelmüller and Mohr (1986). Equal loading of proteins per lane was confirmed by incubating the membranes with Pouceau S (Sigma–Aldrich, Taufkirchen, Germany) prior to the antibody application.

2.8. Analysis of the thylakoid membranes

Four days after the application of 20% PEG solution (or water as control), chloroplasts were isolated from the leaf material by Percoll gradient centrifugation. After lysis, the membranes were pelleted by high speed centrifugation, washed twice with 0.3 M NaCl, the proteins were dissolved in Laemmli loading buffer, and the suspension was incubated for 10 min at 78 °C. After centrifugation, 50 μg of chlorophyll was loaded on each lane. The chlorophyll concentration was determined before protein extraction according to Lichtenthaler and Wellburn (1983). The 40 kDa band was cut off the gel, extracted, digested with trypsin and analyzed by mass spectrometry, as described by Sherameti et al. (2004).

2.9. Northern analysis

Arabidopsis cDNAs (obtained from the Ohio stock center) were used for the hybridization with RNA from Chinese cabbage. The cDNA fragments were excised from the plasmids according to the instruction from the stock center. RNA was isolated with the RNAeasy extraction kit and equal amounts of RNA (35 μg) were

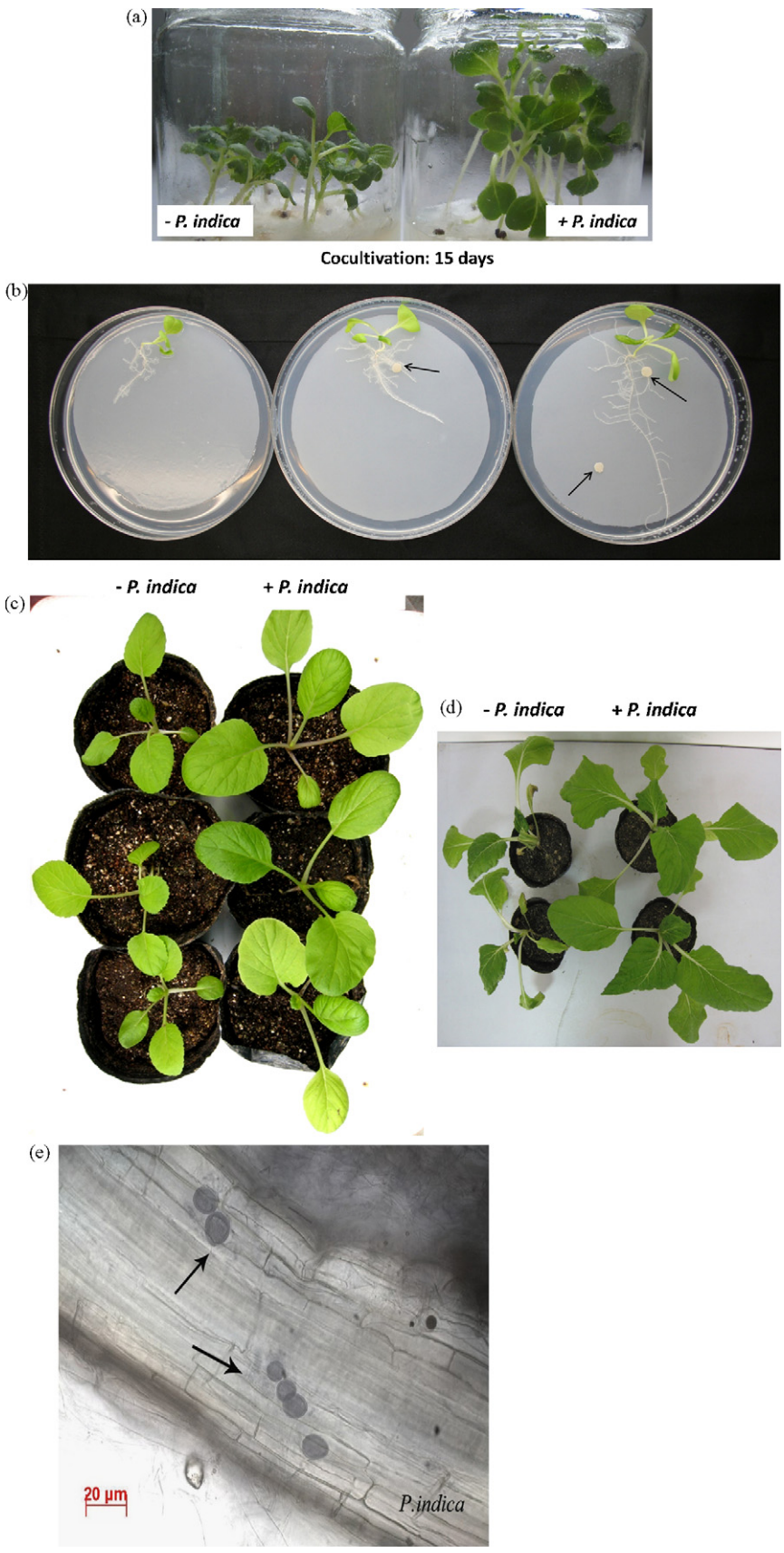

Fig. 1. (A–C) *P. indica* promotes Chinese cabbage growth. (A) The picture shows seedlings kept in jars, which were either co-cultivated with *P. indica* (+ *P. indica*) or mock-treated (− *P. indica*). (B) Sizes of the roots of Chinese cabbage seedlings which were either mock-treated or treated with one or two plaques of *P. indica* (see arrows). The seedlings were co-cultivated with *P. indica* for 7 days in 15 cm-diameter Petri dishes. (C) Colonized and mock-treated plants after transfer to soil for 15 days. (D) Colonized and mock-treated plants after transfer to soil for 20–30 days. For this picture, uncolonized (left) and colonized (right) plants of the same size and weight were chosen and treated once with 200 ml of PEG solution, 4 days before the picture was taken. (E) Spores in the roots. For details cf. Section 2.

separated on a formaldehyde gel. Hybridization occurred at 61 °C for 42 h. Each filter also contained RNA from *Arabidopsis* to confirm that the size of the hybridizing signal corresponds to that of Chinese cabbage RNA. Equal RNA loading was confirmed by hybridization to an actin DNA fragment, which was amplified with the primer pairs described above.

2.10. Statistics

Samples were evaluated with a two sample *t*-test (± *P. indica*, ± drought) and ANOVA analyses (comparison of all data sets). All experiments were repeated three times (RNA and protein data) or four times (enzyme activities and color pictures).

3. Results

3.1. Root colonization stimulates biomass production of Chinese cabbage plants

Growth of Chinese cabbage seedlings, co-cultivated with *P. indica* in jars for 15 days, was strongly promoted compared to mock-treated control seedlings (Fig. 1A). Also, the development of the main and lateral roots was strongly promoted by the fungus (Fig. 1B). After 15 days on soil, the effects were even stronger (Fig. 1C). We observed a 38 ± 5% (n = 20) increase in the root fresh weight and a 46 ± 6% (n = 20) increase in leaf fresh weight. Microscopic analyses revealed that the degree of root colonization was quite high. Spores were detected in almost all areas of the roots, and most of the fungal hyphae could be found in and in the vicinity of the lateral roots (Fig. 1E). Although different plant species are difficult to compare, we determined the amount of the cDNA, generated from RNA of colonized roots, for the translation elongation factor 1 from *P. indica* with the amounts of the *actin* cDNA from Chinese cabbage and *Arabidopsis*. The *Pitef1*/*actin* ratio was at least 5 times higher for Chinese cabbage compared to *Arabidopsis*, which roughly reflects the ratio of spores/lateral root determined under the microscope (7–10 times more spores in Chinese cabbage roots compared to *Arabidopsis* roots). The massive increase in the biomass of the aerial parts of the plant indicates that there must be efficient information flow from the colonized roots to the leaves.

3.2. Chinese cabbage plants co-cultivated with P. indica are more drought-tolerant than the uncolonized control

Due to the fast growth and enormous biomass production of the Chinese cabbage leaves, the plants are quite sensitive to drought, which is a severe problem in agriculture. Therefore, we exposed Chinese cabbage to drought stress. After 4 weeks on soil, colonized and uncolonized Chinese cabbage plants were treated once with 200 ml of a 20% PEG solution (pot size: 10 cm height and 10 cm diameter) and further development of the plants was followed over the next 4 days. After 4 days, PEG-treated plants were severely suffering from drought stress, while no visible stress phenomena could be detected in drought-exposed colonized plants (Fig. 1D).

3.3. P. indica decreases the accumulation of malondialdeyde, a biomarker of oxidative stress in cells

Reactive oxygen species (ROS) degrade polyunsaturated lipids, thereby forming malondialdehyde (MDA, Pryor et al., 1975). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress (Moore and Roberts, 1998; Del Rio et al., 2005). PEG application strongly promoted MDA accumulation in the leaves and the amount of MDA is reduced in colonized plants (Fig. 2). This suggests that the fungus prevents ROS formation by retarding the degradation of polyunsaturated lipids.

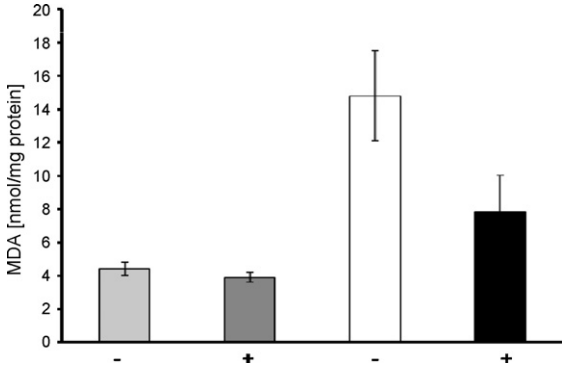

Fig. 2. *P. indica* inhibits MDA accumulation in the leaves of PEG-treated Chinese cabbage plants. Chinese cabbage plants were either treated with 200 ml of water (grey bars) or with 200 ml of PEG solution (black and white bars). –, +: without, with *P. indica*. The measurements were taken 24 h after the PEG/water treatment. Bars represent SEs, based on 4 independent experiments.

3.4. Antioxidant enzyme activities are upregulated in colonized drought-exposed leaves

Several reports have demonstrated that antioxidant enzyme activities are crucial for *P. indica*-induced resistance against abiotic stress (Baltruschat et al., 2008, Vadassery et al., 2009b). Therefore, we tested whether *P. indica* influences the enzyme activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) after exposure of the plants to drought. One day after the PEG application, the SOD activity in the leaves of colonized plants was higher than the activity of all other controls (no PEG treatment, no fungus) (Fig. 3A). A similar effect was observed for the POD activity (Fig. 3B). In contrast, the CAT activity was stimulated in colonized and uncolonized leaves after PEG application, although the stimulatory effect was higher in the presence of the fungus (Fig. 3C). We conclude that *P. indica* stimulates antioxidant enzyme activities in the leaves of PEG-treated Chinese cabbage plants, although the effects for the three enzymes tested were quite different.

3.5. P. indica promotes the expression of drought-related genes after PEG treatment

We have previously demonstrated that a large number of drought-induced genes are more quickly and strongly upregulated in drought-exposed *Arabidopsis* leaves when the roots are colonized by *P. indica* (Sherameti et al., 2008a). Using *Arabidopsis* cDNA probes for drought-related genes, we performed a similar experiment with Chinese cabbage plants exposed to PEG treatment. RNA was isolated from the leaves of colonized and uncolonized Chinese cabbage plants 0, 8, 16 and 24 h after PEG treatment. Fig. 4 demonstrates that the mRNA levels for the transcription factors DREB2A and ANAC072, as well as for the drought-related proteins CBL1 and RD29A, responded faster and more strongly to the PEG treatment in colonized plants compared to the uncolonized controls. This confirms our previous results obtained with *Arabidopsis* seedlings and extends them to adult Chinese cabbage plants grown on soil.

3.6. P. indica retards the breakdown of plastid functions after PEG treatment

The PEG treatment has a strong effect on photosynthesis (Flexas et al., 2004; Ramachandra-Reddy et al., 2004, Sherameti et al., 2008a). The *F* variable/*F* maximum (*Fv*/*Fm*) values decreased in

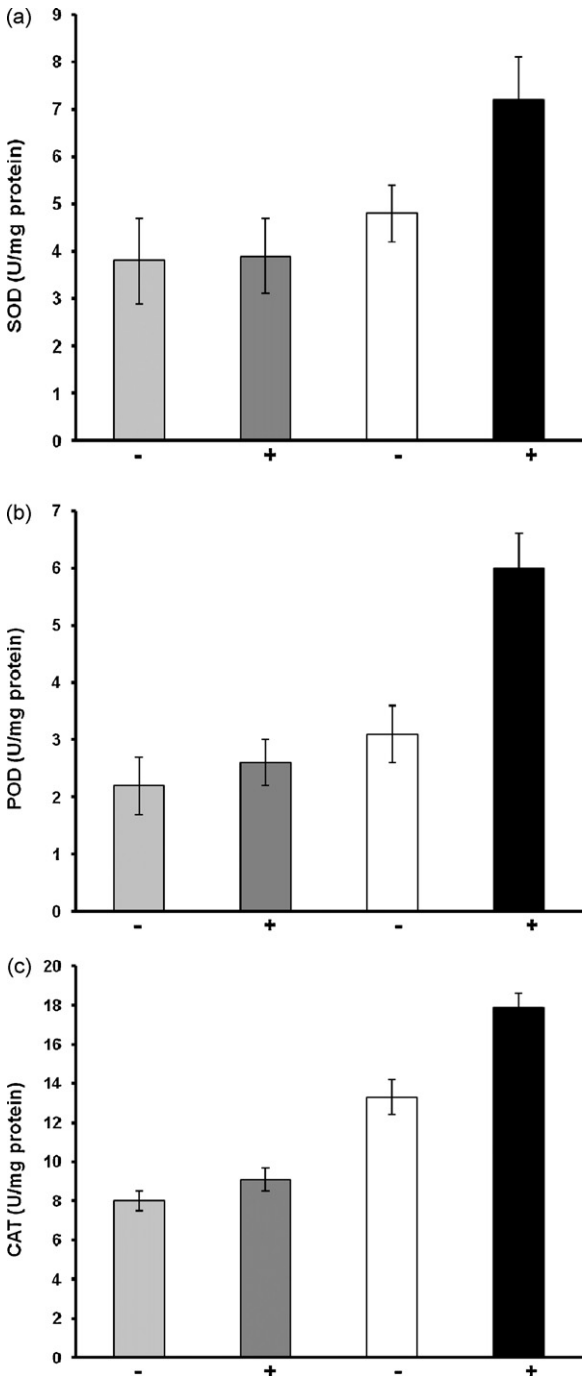

Fig. 3. *P. indica* stimulates antioxidant enzyme activities in the leaves of PEG-treated Chinese cabbage plants. The measurements were taken 24 h after the PEG/water treatment. For details, cf. legend to Fig. 2. (A–C) SOD, POD and CAT activities. Bars represent SEs, based on 4 independent experiments.

the uncolonized, dark-adapted controls within the first 4 days after exposure to drought (from $t_{0d} = 0.82$ to $t_{4d} = 0.61$), while no significant difference was observed for the dark-adapted colonized plants (from $t_{0d} = 0.82$ to $t_{4d} = 0.79$) (Fig. 5A). *Fv*/*Fm* values around 0.83, measured after dark adaptation, reflect the potential fluorescence quantum efficiency of photosystem II and are sensitive indicators of plant photosynthesis performance (Björkman and Demming, 1987). Values lower than 0.83 indicate that plants are exposed to stress (Maxwell and Johnson, 2000). This clearly demonstrates that root-colonized plants suffer less from drought stress than the uncolonized controls.

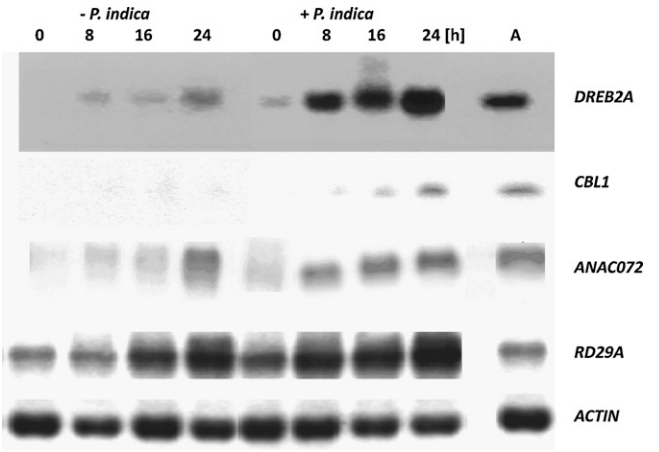

Fig. 4. mRNA levels for drought-related genes (and *ACTIN* as loading control) in the leaves of root-colonized (+ *P. indica*) or mock-treated (− *P. indica*) Chinese cabbage leaves, 0, 8, 16 and 24 h after the PEG treatment. A, RNA from Arabidopsis leaves was used as control. Representative Northern blots from 3 independent experiments are shown.

In addition, 4 days after PEG application, the total chlorophyll level decreased by more than 50% in uncolonized plants, whereas *P. indica*- colonized plants showed only a minor decrease in the total chlorophyll content (Fig. 5B). Furthermore, the decrease in the protein levels of representative components of the thylakoid membrane and of enzymes located in the plastid stroma in PEG-treated plants was retarded in the presence of *P. indica* (Fig. 5C). We analyzed the protein levels of representative components of the photosystem II (D1, 33- and 23-kDa proteins), the photosystem I (PsaA, PsaD, PsaF), the cytochrome-*b6*/*f*-complex (PetC, Rieske protein), the small subunit of ribulose-1,5-bisphosphate (RbcS), nitrate reductase (NR), ferredoxin (Fd) and of a regulatory protein involved in iron-sulfur biosynthesis (Hcf101; Stöckel and Oelmüller, 2004). Analysis of the expression levels of four nuclear and two plastid-encoded genes for thylakoid proteins confirmed that this regulation occurs at the level of transcription (Fig. 5D). The best hybridization signals with the *Arabidopsis* probes on Chinese cabbage RNA filters were obtained for genes encoding the 33-kDa protein of photosystem II, plastocyanin, the subunit III of photosystem I (PsaF), and the subunit δ of the plastid ATP synthase (AtpD), as well as for the two major core proteins of the photosystems II and I, PsbA and PsaA. Therefore, root-colonized Chinese cabbage plants are better protected against drought stress.

3.7. *The plastid-localized calcium-sensing receptor CAS is upregulated in colonized leaves exposed to drought*

While analyzing the protein composition of the thylakoid membrane, we noted that a protein of approximately 40 kDa was upregulated in *P. indica*- colonized PEG-treated Chinese cabbage leaves (Fig. 6A). The protein was extracted from the gel and analyzed by mass spectrometry. Two fragments corresponded to VFQVVGDALK and AQEAIQSSGFDSEPVFNAAK. A data bank search revealed that both fragments show an almost complete match to the CAS protein from various organisms (cf. Nomura et al., 2008; Weinl et al., 2008). Thus, it is likely that the plastid-localized CAS protein is a target of *P. indica* in drought-stressed Chinese cabbage leaves. Northern analysis with a cDNA probe from *Arabidopsis* demonstrated that the mRNA level follows the pattern observed on the protein level (Fig. 6B). This suggests that the regulation occurs at the level of transcription.

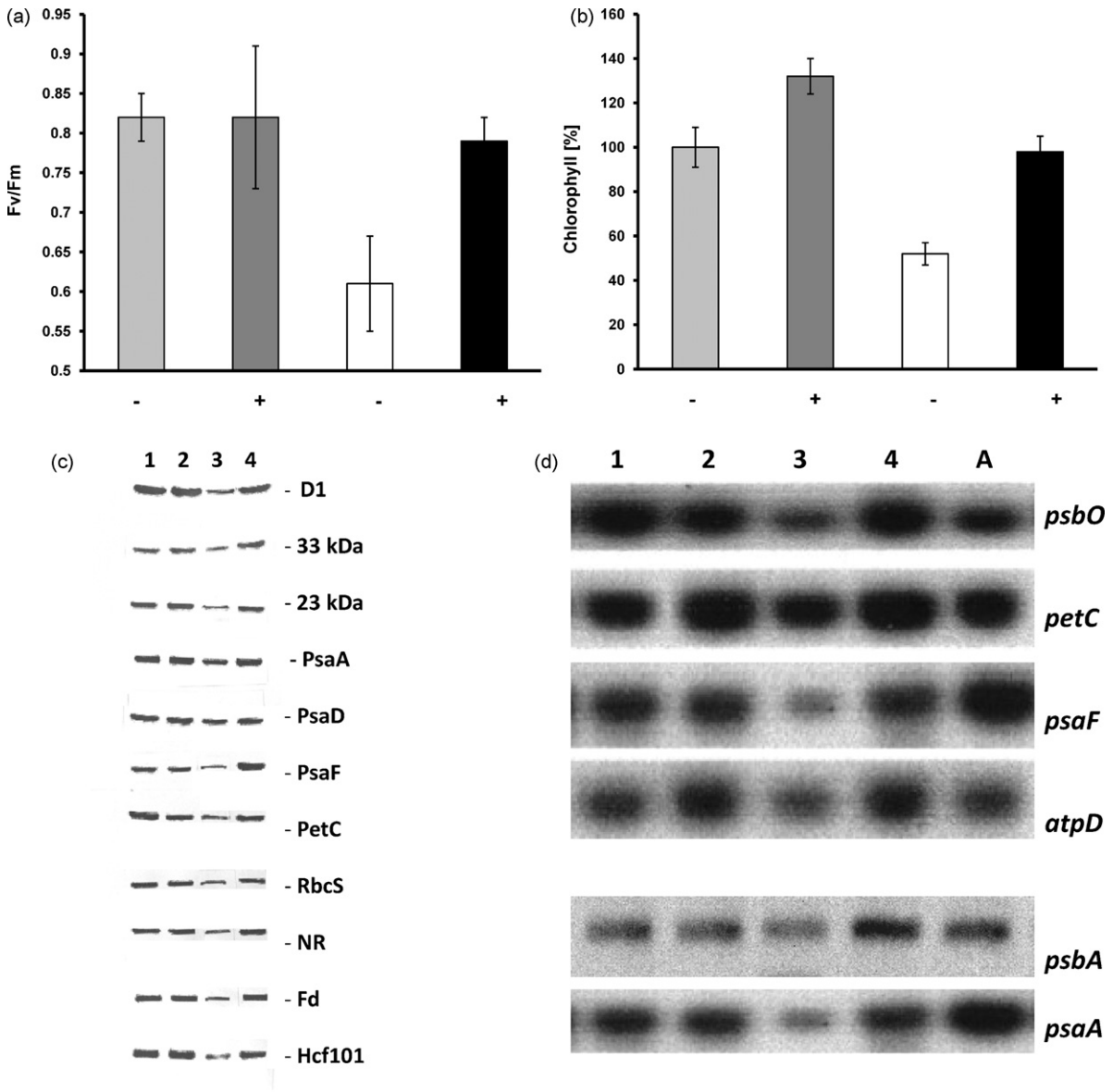

Fig. 5. The effect of *P. indica* on photosynthesis and plastid proteins during drought stress. Chinese cabbage plants were either treated with 200 ml of water (grey bars in figures with bar graphs and lanes 1 and 2 in gel pictures) or with 200 ml of 20% PEG solution (black and white bars in figures with bar graphs and lanes 3 and 4 in gel pictures). (−) and lanes 1 and 3: plants without *P. indica*; (+) and lanes 2 and 4: plants co-cultivated with *P. indica*. (A) Fv/Fm values 24 h after the PEG/water treatment. Bars represent SEs, based on 16 independent experiments. (B) Total chlorophyll content 96 h after the PEG/water treatment. Bars represent SEs, based on 16 independent experiments. The chlorophyll content in the leaves of uncolonized water-treated plants was taken as 100% and the other values are expressed relative to it. (C) Western analyses for plastid proteins, 96 h after PEG/water treatment. D1, reaction center protein of photosystem II; 33 and 23 kDa, two lumenal proteins of photosystem II; PsaA, reaction center protein of photosystem I; PsaD, PsaF, subunits II and III of photosystem I; PetC, the Rieske Fe/S-protein of the cytochrome-*b6*/*f*-complex; RbcS, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; NR, nitrate reductase; Fd, ferredoxin; Hcf101, regulatory protein for photosystem I assembly. (D) Northern analyses for representative nuclear-encoded (*psbO*, *petC*, *psaF*, *atpD*) and plastid-encoded (*psbA*, *psaA*) genes. A, RNA from Arabidopsis leaves were used as control. *psbO*, 33 kDa protein of photosystem II; *petC*, the Rieske protein of the cytochrome-*b6*/*f*-complex; *psaF*; subunit III of photosystem I; *atpD*, subunit δ of the ATP synthase; *psbA*, D1 protein; *psaA*, reaction center protein of photosystem I.

4. Discussion

4.1. Chinese cabbage is a good host for P. indica

We noted that *P. indica* strongly interacted with the roots of Chinese cabbage, resulting in efficient colonization (Fig. 1). Hyphae and spores were detected around the roots and root hair, in the extracellular space and within root cells (Fig. 1D). Co-cultivation of both organisms resulted in a rapid increase in root and shoot biomass (Fig. 1A–C). The growth-promoting effect remained visible after transfer of the seedlings to soil (Fig. 1C). Closer inspection of the root of the adult plants revealed that the hyphae continued to propagate in the soil (data not shown). Therefore, once colonized, the symbiosis remains stable and provides the basis for long-term benefits for the plant. Rough estimations suggest that the fungal hyphae propagate much better in Chinese cabbage roots than in *Arabidopsis* roots. Because of the enormous ecological and agricultural importance of Chinese cabbage, we investigated this interaction in greater detail.

4.2. P. indica confers drought tolerance by activating the antioxidant enzyme systems

Accumulation of MDA, a biomarker of oxidative stress, in PEG-treated Chinese cabbage clearly demonstrated that the plants were

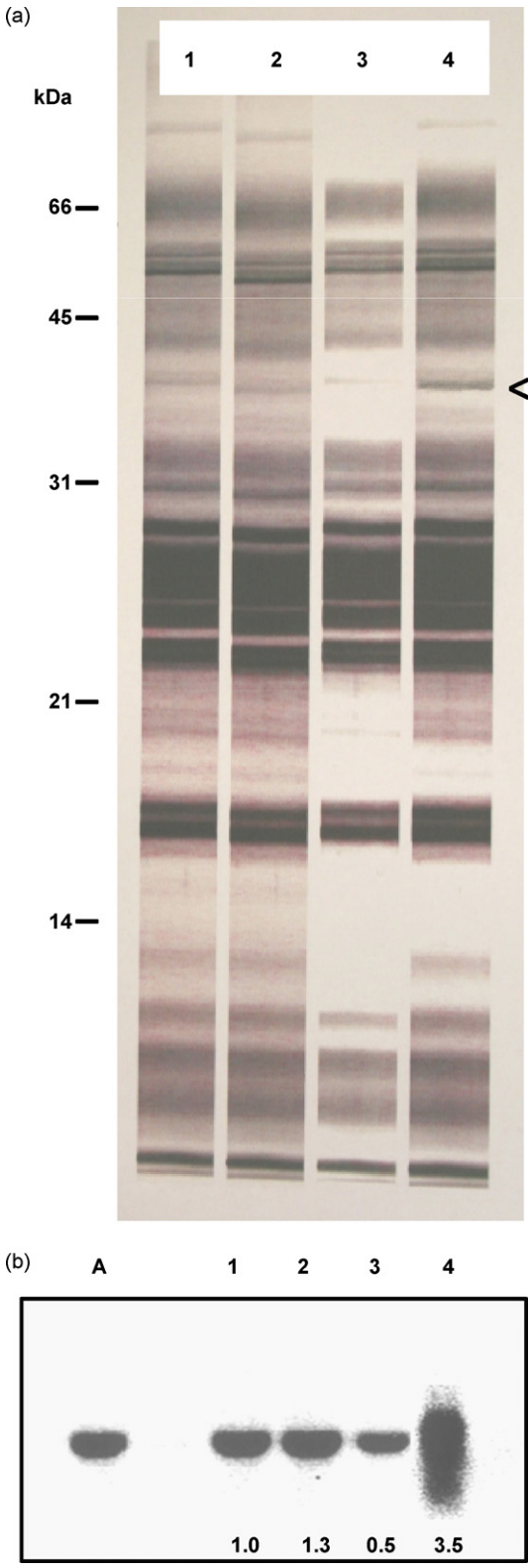

Fig. 6. The plastid-localized CAS is a target of *P. indica* in PEG-treated Chinese cabbage plants. (A) Protein pattern of thylakoid membranes isolated from chloroplasts of Chinese cabbage leaves which were either treated with PEG solution 96 h before plastid isolation (lanes 3 and 4) or mock-treated with water (lanes 1 and 2). Lanes 1 and 3: uncolonized plants; lanes 3 and 4; plants co-cultivated with *P. indica* for 30 days on soil. Proteins corresponding to 50 μg of chlorophyll was loaded per lane. The 40 kDa band that is upregulated in lane 4 is marked. (B) Northern analysis for the *CAS* mRNA. RNA was isolated 96 h after the PEG/water treatment. A, RNA from Arabidopsis leaves was used as control. The numbers under the hybridization signals refer to the amounts of the mRNAs, relative to the level in lane 1.

exposed to stress (Fig. 2). The overall level of MDA was lower in *P. indica*-colonized plants, and thus the fungus could partially counteract this stress response. MDA is mainly formed by the ROS-induced degradation of polyunsaturated lipids (Pryor et al., 1975; Del Rio et al., 2005). *P. indica* could prevent or retard the degradation of these lipids by preventing excess ROS formation under stress conditions. This confirms the results obtained for the antioxidant enzyme activities that become activated in the leaves of colonized plants (Fig. 3A–C). We focused on three classes of enzymes: the SODs catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. In higher plants, SOD isozymes are present in different cell compartments. Mn-SOD is present in mitochondria and peroxisomes, Fe-SOD mainly in chloroplasts, and Cu/Zn-SOD in cytosol, chloroplasts, peroxisomes and the apoplast (Corpas et al., 2001, 2006). In green leaves, the majority of the SOD activity is present in plastids, which also demonstrates the important role of this organelle for the *P. indica*-induced drought tolerance. CATs, peroxisomal enzymes (Alberts et al., 2002), catalyze the decomposition of hydrogen peroxide to water and oxygen (Chelikani et al., 2004). Apparently, antioxidant enzyme activities in the chloroplast-associated peroxisomes are also important targets of *P. indica* in Chinese cabbage leaves.

PODs are a large family of enzymes that detoxify hydrogen peroxide, organic hydroperoxides or lipid peroxides to generate alcohols. PODs contain a heme cofactor in their active sites that is synthesized in the plastid. Heme is also coupled to the iron homeostasis, which may play an important role in plant/microbe interaction (Briat et al., 2007; Stöckel et al., submitted for publication). In addition, PODs contain redox-active cysteine residues, which directly measure the redox potential in the cell or organelle. The most important organelle in a green leaf that controls the redox potential in the cell is the plastid (Mühlenbock et al., 2008; Bräutigam et al., 2009). Whether the fungus interferes with the iron homeostasis and redox potential of the cell and whether this contributes to stress tolerance remains to be investigated. Most of the antioxidant enzymes are regulated posttranslationally in response to oxidative stress. Therefore, we determined the enzyme activities rather than the expression levels of the genes in response to *P. indica*. The data are consistent with previous observations that activation of the antioxidant enzyme systems is a major target of the fungus in leaves (Baltruschat et al., 2008; Vadassery et al., 2009b). In the leaves of colonized *Arabidopsis* plants, monodehydroascorbate reductase and dehydroascorbate reductase, two enzymes of the ascorbate-glutathione cycle that maintain ascorbate in its reduced state, were upregulated. Growth and seed production were not promoted by the fungus in *mdar2* and *dhar5* T-DNA insertion lines. Thus, MDAR2 and DHAR5 are crucial for producing sufficient ascorbate to maintain the interaction between *P. indica* and *Arabidopsis* in a mutualistic state (Vadassery et al., 2009b). It remained to be determined how the information is transferred from the roots to the leaves.

We have previously demonstrated that the mRNA levels for 9 drought-related genes are upregulated in the leaves of *P. indica*-colonized *Arabidopsis* plants (Sherameti et al., 2008a). In addition to transcription factor genes, which stir the proper response of the plant to drought stress (Shinozaki et al., 2003, 2007; Yamaguchi-Shinozaki et al., 2006), *EARLY RESPONSE TO DEHYDRATION (ERD)1* was also strongly regulated by the fungus. ERD1 is a plastid-localized Clp protease regulatory subunit (Nakashima et al., 1997). Here, we show that four of these genes are also upregulated in Chinese cabbage leaves when they are exposed to drought. Although *ERD1* is not included in this study because the heterologous

Arabidopsis probe did not yield reliable data, our results suggest that drought tolerance in the two species might be achieved by similar mechanisms, for instance by establishing reducing conditions in the leaf cells.

4.3. Major targets of P. indica in Chinese cabbage leaves are located in the plastids

Plastids are the major targets of the PEG treatment in Chinese cabbage leaves. In addition to a reduction in photosynthetic efficiency, not only the pigments, but also representative proteins of the photosynthetic machinery and the biosynthetic pathways in the stroma were affected by the PEG treatment (Fig. 5A–D). Interestingly, both nuclear and plastid gene expression were down-regulated (Fig. 5D), indicating that drought stress causes a more general lesion of plastid functions. It is likely that *P. indica* does not target specific photosynthesis genes or proteins to establish drought tolerance, but creates an atmosphere in the cell that prevents a general degradation of plastid functions. Drought stress affects plastid protein degradation to different extents, which is not surprising considering the different stabilities of the thylakoid proteins (cf. Palomares et al., 1993). It is a task for future studies to understand the molecular basis of how the fungus can prevent plastid protein degradation.

4.4. The role of CAS in conferring drought tolerance in Chinese cabbage

Interestingly, the plastid-localized CAS protein appears to be a major target of *P. indica* in the chloroplasts under drought stress (Fig. 6A and B). It has been proposed that the protein – although located in the plastids – controls cytosolic calcium transients and thus stomatal closure (Nomura et al., 2008; Weinl et al., 2008). The CAS protein and *CAS* mRNA levels did not increase after exposure of uncolonized Chinese cabbage to drought stress, although this could ensure better protection of the plant against drought because the stomata could be closed more efficiently. One reason for this observation could be that the severe damage to the plastids after the PEG treatment does not allow a proper regulation of plastid functions. The presence of *P. indica* creates a cellular environment that protects the plastids against severe damage and this allows a proper regulation of *CAS* gene expression. Since drought-stressed *P. indica*-colonized plants clearly showed higher *CAS* mRNA and CAS protein levels in the leaves, it is likely that the fungus counteracts the drought stress by elevating cytoplasmic calcium transients which finally results in stomata closure in the guard cells (Nomura et al., 2008). However, CAS does not only stimulate cytoplasmic calcium elevation in the guard cells, but also in all chloroplast-containing cells of the leaves. This could lead to the activation of a large battery of calcium-induced cellular responses, including plant defense against pathogens. It remains to be determined whether CAS is involved in other stress avoidance responses. The availability of efficient transformation systems for Chinese cabbage will allow us to investigate the role of the CAS protein in greater detail, and to understand the role of this protein in *P. indica*-mediated stress tolerance.

Acknowledgements

This work was supported by a grant (Z3080241) of the Zhejiang provincial natural foundation of China to B.L. and the SFB 605 to R.O.

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