Bacterial endophytes contribute to abiotic stress adaptation in pepper plants (*Capsicum annuum* L.)

A.H. Sziderics, F. Rasche, F. Trognitz, A. Sessitsch, and E. Wilhelm

Abstract: Endophytes are nonpathogenic plant-associated bacteria that can play an important role in plant vitality and may confer resistance to abiotic or biotic stress. The effects of 5 endophytic bacterial strains isolated from pepper plants showing 1-aminocyclopropane-1-carboxylate deaminase activity were studied in sweet pepper under in vitro conditions. Four of the strains tested showed production of indole acetic acid. Plant growth, osmotic potential, free proline content, and gene expression were monitored in leaves and roots under control and mild osmotic stress conditions. All indole acetate producers promoted growth in *Capsicum annuum* L. 'Ziegenhorn Bello', from which they were isolated. Osmotic stress caused an increase in the content of free proline in the leaves of both inoculated and noninoculated plants. Inoculated control plants also revealed higher proline levels in comparison with noninoculated control plants. Differential gene expression patterns of *CaACCO*, *CaLTPI*, *CaSAR82A*, and putative *P5CR* and *P5CS* genes during moderate stress were observed, depending on the bacterium applied. Inoculation with 2 bacterial strains, EZB4 and EZB8 (*Arthrobacter* sp. and *Bacillus* sp., respectively), resulted in a significantly reduced upregulation or even downregulation of the stress-inducible genes *CaACCO* and *CaLTPI*, as compared with the gene expression in noninoculated plants. This indicates that both strains reduced abiotic stress in pepper under the conditions tested.

Key words: pepper, endophytes, ACC deaminase, IAA, abiotic stress, gene expression.

Résumé : Les endophytes sont des bactéries non pathogènes associées aux plantes qui peuvent jouer un rôle important dans la viabilité de la plante et peuvent conférer une résistance à des stress abiotiques ou biotiques. Les effets de cinq souches bactériennes endophytes isolées de plants de poivrons qui démontrent une activité 1-aminocyclopropane-1-carboxylate désaminase ont été étudiés chez le poivron doux in vitro. Quatre des souches testées produisaient de l'acide indole-acétique. La croissance des plants, le potentiel osmotique, le contenu en proline libre et l'expression génique ont été examinés dans les feuilles et les racines placées en conditions de stress osmotique faible ou contrôle. Toutes les souches qui produisaient de l'acide indole-acétique étaient promotrices de la croissance de *Capsicum annuum* L. 'Ziegenhorn Bello' de la quelle elles avaient été isolées. Le stress osmotique a causé une augmentation du contenu en proline libre dans les feuilles de plants inoculés ou non. Les plants inoculés contrôles ont aussi révélé un contenu élevé en proline libre comparativement aux plants contrôles non inoculés. Lors d'un stress modéré, des patrons différentiels d'expression des gènes *CaACCO*, *CaLTPI*, *CaSAR82A* ainsi que des gènes présumés *P5CR* et *P5CS* ont été observés selon la bactérie inoculée. L'inoculation de deux souches bactériennes, EZB4 et EZB8 (*Arthrobacter* sp. et *Bacillus* sp.) a résulté en une réduction significative de l'activation, voire une inhibition, de l'expression des gènes *CaACCO* et *CaLTPI* inductibles par le stress, comparativement aux plants non inoculés. Ceci indique que les deux souches réduisent le stress abiotique chez le poivron dans les conditions testées.

Mots-clés : poivron, endophytes, ACC désaminase, IAA, stress abiotique, expression génique.

[Traduit par la Rédaction]

Introduction

Bacterial endophytes include "bacteria, which for all or part of their life cycle invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease" (Wilson 1995). Endophytes colonize a similar ecological niche as plant pathogens and may gain entry into plants by penetrating root hair cells (Huang 1986) or by producing cell-wall-degrading enzymes (Huang 1986; Quadt-Hallmann et al. 1997). Endophytes mainly colonize intercellular spaces of plants as well as vascular tissues and may systematically colonize plant tissues (Compant et al. 2005).

A high number of bacterial species has been isolated from plant tissues, such as seeds, roots, stems, and leaves (Hallmann et al. 1997; Sturz et al. 1997; Surette et al. 2003), and cultivation-independent analysis showed that a high number of unculturable species also colonize plants endophytically (Chelius and Triplett 2001; Idris et al. 2004). It has been demonstrated that plant stress significantly affects endophyte communities, most probably because of plant physiological changes (Reiter et al. 2002; Sessitsch et al. 2002; Rasche et al. 2006*a*, 2006*b*). Various endophytic bacteria have been shown to have several beneficial effects on their host plant, and the mechanisms involved are probably similar to those described for rhizosphere bacteria. Plant

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A.H. Sziderics, F. Rasche, F. Trognitz, A. Sessitsch,[1] and E. Wilhelm. Austrian Research Centers GmbH, Department of Bioresources, Seibersdorf A-2444, Austria.

[1]Corresponding author (e-mail: angela.sessitsch@arcs.ac.at).

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growth promotion may be achieved through the production of plant growth enhancing substances such as indole acetic acid (IAA) (Beyeler et al. 1999) or cytokinins (Timmusk et al. 1999). Beneficial effects on plant growth may also be achieved by improved nutrient aquisition, including nitrogen fixation (Mirza et al. 2001; Reiter et al. 2003; Vessey 2003). Endophytes may produce the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Campbell and Thompson 1996; Shah et al. 1998). This enzyme has no function in bacteria but cleaves ACC, the precursor of ethylene in plants, and thus modulates ethylene levels, which contributes to plant growth promotion (Glick et al. 1997; Burd et al. 1998; Grichko and Glick 2001; Mayak et al. 2004*a*, 2004*b*).

Almost all biotic and abiotic stress conditions elicit ethylene synthesis in plants (Bleecker and Kende 2000). This gaseous plant hormone acts as a signalling molecule and is involved in important physiological processes such as seed germination, plant growth, fruit ripening, senescence, and pathogen defense (Abeles et al. 1992). High levels of ethylene are usually deleterious to plant growth and health, except for fruit ripening and the initiation of lateral root growth (Czarny et al. 2006).

Ethylene is formed from methionine via *S*-adenosyl-L-methionine, which is converted into ACC. This cyclic non-protein amino acid is converted to ethylene, catalyzed by the enzyme ACC oxidase (Bleecker and Kende 2000). It has been demonstrated that rhizosphere bacteria that exhibit ACC deaminase activity improve plant growth and reduce plant stress (Burd et al. 1998; Grichko and Glick 2001; Mayak et al. 2004*a*, 2004*b*). However, the interactions between endophytes, plants, and stress are not well understood, and very little information on the effect of beneficial bacteria on plant gene expression is available.

The aim of this study was to test the growth-promoting properties of endophytes that were isolated from stems of pepper plants. The potential of endophytes to reduce osmotic stress was evaluated by analyzing the osmotic potential of roots and the contents of free proline in leaves, as well as by quantifying the expression of candidate genes.

Materials and methods

Plant material and growth conditions

Seeds of the sweet pepper *Capsicum annuum* L. 'Ziegenhorn Bello' (ZB; Austrosaat, Vienna, Austria) were surface sterilized by a short rinse with 70% (*v*/*v*) ethanol and subsequent incubation in a 6% (*v*/*v*) sodium hypochlorite solution for 10 min followed by 5 rinses with sterile water. The seeds were then placed on hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 2% (*v*/*v*) sucrose for germination. When seedlings had developed 4 leaves, they were transferred to Magenta GA-7 vessels (Sigma-Aldrich, St. Louis, Missouri, USA) containing clay granules amended with 40 mL of MS medium containing 1% (*v*/*v*) sucrose. Nine plantlets were placed in a single box.

Analysis of ACC deaminase activity

Bacterial strains, which were previously isolated from shoots of ZB according to the method described by Rasche et al. (2006*b*), were tested for ACC deaminase production. Strains were grown on Brown and Dilworth minimal medium (Brown and Dilworth 1975) containing 0.7 $g \cdot L^{-1}$ ACC as the sole nitrogen source. Brown and Dilworth plates containing 0.7 $g \cdot L^{-1}$ NH_4Cl were used as positive controls, and Brown and Dilworth plates without any nitrogen source were used as negative controls. An ACC-deaminase-producing bacterium (*Burkholderia phytofirmans* PsJN; NCBI accession No. AY497470) and a nonproducing strain (*Methylobacterium* sp. iEII1) were used as positive and negative controls, respectively. Growth of the bacteria was monitored after 7 days of incubation at 30 °C.

Analysis of indole acetate production

IAA production of the bacteria was tested according to a modified method of Sawar and Kremer (1995). Bacteria were grown on half-strength tryptic soy broth agar (Merck, Darmstadt, Germany) at 30 °C. Some cell material of 24 h cultures was added to 5 mL of growth medium containing 5 $g \cdot L^{-1}$ glucose, 0.025 $g \cdot L^{-1}$ yeast extract, and 0.204 $g \cdot L^{-1}$ L-tryptophan and was incubated in the dark for 72 h at 25 °C and 180 $r \cdot min^{-1}$. One uninoculated culture tube was kept as a negative control. For qualitative determination of IAA production, 1.5 mL of the bacterial suspension was centrifuged at 8000*g* at 4 °C for 10 min. Cell-free supernatant (900 μL) was mixed with 600 μL of Salkowski reagent (0.5 $mol \cdot L^{-1}$ $FeCl_3$ and 35% (*v*/*v*) perchloric acid in a ratio of 1:50). Following reaction for 30 min in the dark, a pink to purple colour indicated IAA production.

Inoculation with endophytic bacteria and osmotic stress treatment

Plants were inoculated 8 days after transfer to Magenta boxes by adding 9 mL of MS medium with 2% (*v*/*v*) sucrose and 1 mL of 10% (*v*/*v*) tryptic soy broth (Merck) containing 10^6–10^7 colony-forming units of bacteria. In total, 5 strains (listed in Table 1) were tested. The control without bacterial inoculation was treated accordingly.

Three weeks after inoculation at the 6–8 leaf stage of the plantlets, 10 mL of an MS solution containing 45% (*v*/*v*) polyethylene glycol (PEG) 6000 (Fluka, Buchs, Switzerland) and 2% (*v*/*v*) sucrose was added to 2 boxes per strain to simulate mild osmotic stress. Unstressed control plants received MS medium without PEG 6000.

Biomass

Three days after the stress treatment, 15 plants per treatment were harvested and separated into roots, stems, and leaves. After fresh mass determination, tissues were immediately frozen in liquid nitrogen and stored at –80 °C until further analysis.

Osmotic potential and pH

The liquid medium osmotic potential, Ψ, was measured according to Prewein et al. (2004). The freezing point depression of the solutes, which was directly proportional to the osmolality of the liquid samples (solute concentration expressed in $mOsm \cdot kg^{-1}$), was determined using a digital micro-osmometer (VOGEL, Giessen, Germany). A multiplication factor (–2.4789) was used to convert osmolality ($Osm \cdot kg^{-1}$) into osmotic potential (Ψ, MPa) at 25 °C (Pre-

Table 1. Identity as analyzed by sequence analysis of the partial 16S rRNA gene by Rasche et al. (2006*a*) and potential plant growth-promoting activities of endophytic isolates obtained from sweet pepper *Capsicum annum* L. 'Ziegenhorn Bello'.

Strain	Closest match (NCBI acc. No.); % homology	Phylogenetic group	Plant growth-promoting activities
EZB4	*Arthrobacter* sp. 19503 (AJ315071); 99	High-G+C Gram positive	ACCD, IAA
EZB8	*Bacillus* sp. TW4 (AB126771); 100	Firmicutes	ACCD, IAA
EZB18	*Arthrobacter tecti* (AJ639829); 98	High-G+C Gram positive	ACCD, IAA
EZB20	*Arthrobacter* sp. 19503 (AJ315071); 99	High-G+C Gram positive	ACCD, IAA
EZB22	*Microbacterium* sp. R1 (AY974047); 99	High-G+C Gram positive	ACCD

Note: NCBI, National Center for Biotechnology Information; ACCD, 1-aminocyclopropane-1-carboxylate deaminase; IAA, indole acetic acid.

Table 2. Genes analyzed by real-time reverse transcriptase – polymerase chain reaction.

Code, gene name	Encoded protein	GenBank acc. No., NCBI/TIGR	Forward primer; reverse primer	Product size (bp)
CaLTPI	Lipid transfer protein I	AF208832	5′-TGGTGTCAAGATTCCATTCG-3′; 5′-GCCATTCTCGACCCATCTTA-3′	145
CaSAR82A	Systemic acquired resistance	AF112868	5′-CTGACCCAAGCGATGAATG-3′; 5′-AATAGTCACAACGGCCATGA-3′	145
CaACCO	1-Aminocyclopropane-1-carboxylate oxidase	AJ011109	5′-AGTGGCCTTCAACTCCTCAA-3′; 5′-CCGTCTGTTTGAGCAATCACT-3′	149
Putative *P5CR*	Putative Δ^1-pyrroline-5-carboxylate reductase	CO907770	5′-GTGTCGCAGTTAAGGCCAAT-3′; 5′-ACCAACAGCAGAGGGAGTGT-3′	141
Putative *P5CS*	Putative Δ^1-pyrroline-5-carboxylate synthetase	TC4099	5′-GCTGCTCAACAAGCTGGATA-3′; 5′-AGCAAGCTCCGTCCTCTTTA-3′	147
	Polyubiquitin	AY489050	5′-CACGAGCCTTGCTGATTACA-3′; 5′-GTCAATGGTGTCGGAGCTTT-3′	142
	Actin	AY572427	5′-AGCACCCTGTGCTTCTCACT-3′; 5′-GTACGGCCACTGGCATAAAG-3′	145

wein et al. 2004). pH values of nutrient solutions were measured immediately after harvest, using a pH meter.

For analysis of the root osmotic potential, Ψ, 5 plants were pooled, resulting in 3 replicates per treatment. Roots were immersed in liquid nitrogen and ground to a fine powder and stored at –80 °C. Approximately 500 mg of root tissue was thawed at room temperature and centrifuged twice for 5 min at maximum speed. The osmotic potential of the supernatant was then determined as described above.

Proline in leaves

Three replicates of unstressed and stressed plants inoculated with strains EZB4 and EZB8 (see Table 1), respectively, as well as of the noninoculated treatment, were analyzed. Each replicate consisted of 5 pooled plants. Free proline was extracted from 500 mg of leaves and determined by spectrophotometric analysis at 520 nm according to Bates et al. (1973).

Gene expression

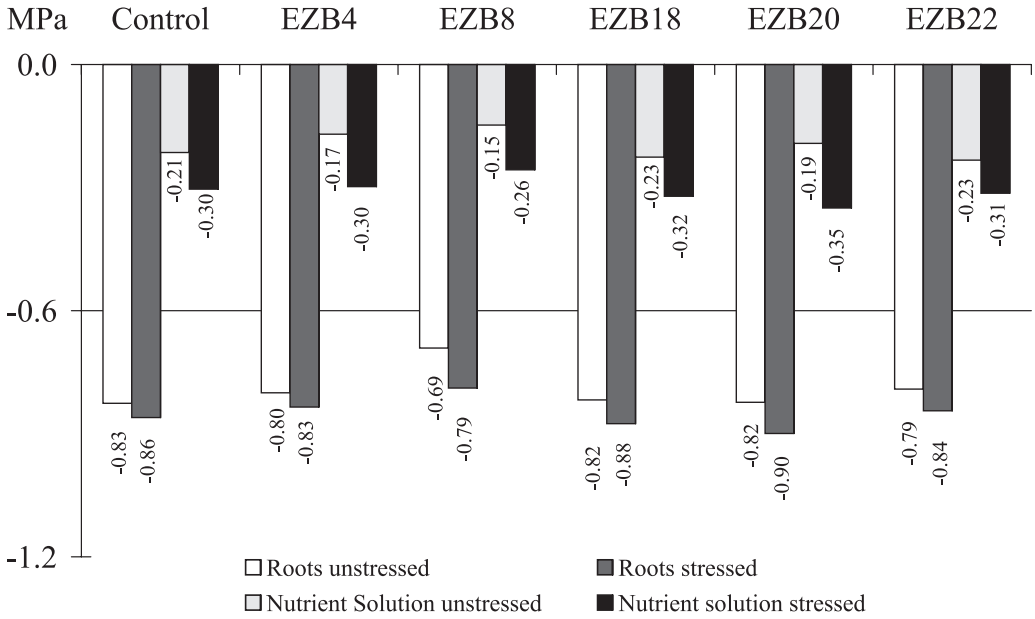
The selected genes, their corresponding accession numbers, and primers used are listed in Table 2. We tested a *Capsicum annuum* nonspecific lipid transfer protein gene (*CaLTPI*) (Jung et al. 2003), a *Capsicum annuum* SAR8.2 gene (*CaSAR82A*) (Lee and Hwang 2003), a *Capsicum annuum* ACC oxidase gene (*CaACCO*) (Garcia-Pineda and Lozoya-Gloria 1999), and 2 putative proline synthesis related genes (Hare and Cress 1997). The 2 putative proline synthesis related genes showed high similarities to genes of tomato and potato encoding Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) and Δ^1-pyrroline-5-carboxylate reductase (*P5CR*).

Gene expression in the leaves and roots of ZB was measured in unstressed and stressed plants, which were inoculated with strains EZB4 and EZB8, respectively. In addition, the noninoculated control was analyzed. Leaves and roots of 5 plants per treatment were pooled, resulting in 3 replicates per treatment. Total RNA was isolated by using the RNeasy™ Plant Mini Kit of Qiagen (Hilden, Germany) as described by the manufacturer. DNA contaminants were removed by using RNase-free DNase (Qiagen, Hilden, Germany) according to the corresponding protocol. All samples were checked by standard agarose gel electrophoresis.

About 0.5 μg of total RNA was transcribed into cDNA according to the protocol of SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, California, USA).

Real-time PCR was carried out in a BioRad iCycler. The reaction mixture contained (in a total volume of 25 μL) 12.5 μL of SYBR Green qPCR Supermix-UDG (Invitrogen, Carlsbad, California, USA), 0.3 $\mu mol \cdot L^{-1}$ of each primer, and 1.0 μL of cDNA. PCR was performed, starting with 2 min at 50 °C and followed by 3 min at 95 °C and 40 cycles of 95 °C for 15 s and 60 °C for 45 s. Melting curves were run immediately after the last cycle to exclude any influence

Fig. 1. Osmotic potential of roots of Ziegenhorn Bello and nutrient solutions (mean values ± SE).



of primer–dimer pairs. Each reaction was performed in triplicates to increase the reproducibility. Cycle numbers at which the fluorescence passed the cycle threshold were further analyzed using the ΔΔcycle threshold method and REST® (Relative Expression Software Tool) (Pfaffl et al. 2002). The housekeeping genes polyubiquitin and actin were analyzed using the BestKeeper™ software (Pfaffl et al. 2004).

Statistical analysis

Data were analyzed using analysis of variance and Student's *t* test. All hypotheses were tested at a 95% confidence interval level. Results of the real-time reverse transcriptase – polymerase chain reaction (RT–PCR) were analyzed using REST® (Pfaffl et al. 2002).

Results and discussion

Biomass

Endophytic bacteria were isolated from pepper plants (Frank Rasche, unpublished data) and analyzed for ACC deaminase production. Five ZB endophytes belonging to the genera *Arthrobacter*, *Bacillus*, and *Microbacterium*, which showed ACC deaminase activity but, which according to their identity, did not suggest any human or plant pathogenicity, were chosen for the present study. All endophytes except strain EZB22 showed IAA production. Besides many effects, this plant hormone may stimulate cell elongation and cell division (Davies 1995).

Selected strains were used to inoculate aseptically grown pepper plants. Three weeks after inoculation, moderate osmotic stress was applied, and after 3 days of stress, plants were harvested to determine whether inoculation had an effect on biomass. Macroscopic symptoms caused by the stress treatment were not visible. Osmotic stress did not affect biomass. All endophytes significantly increased total biomass (Table 3), except for strain EZB22, a bacterium belonging to the genus *Microbacterium*. Only strains EZB4 (*Arthrobacter* sp.) and EZB8 (*Bacillus* sp.) significantly increased root biomass (Table 3). All IAA-producing strains significantly enhanced plant growth already after 3 weeks of cultivation of ZB, indicating that these strains colonized ZB efficiently and that IAA production might have contributed to the observed beneficial effects. Further analysis was performed with strains EZB4 and EZB8, which represent members of the genera *Arthrobacter* and *Bacillus*, respectively.

Table 3. Fresh mass (g) of inoculated and noninoculated pepper plants.

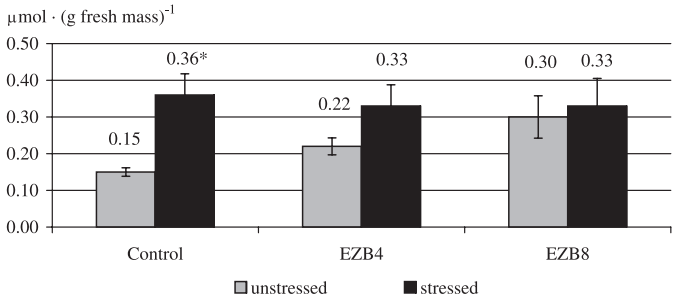
	Ziegenhorn Bello		
Strain	Leaves	Stems	Roots
Control	0.26±0.014	0.11±0.007	0.10±0.008
EZB4	0.33±0.020*	0.20±0.007*	0.13±0.008*
EZB8	0.38±0.023*	0.19±0.012*	0.13±0.011*
EZB18	0.36±0.018*	0.16±0.009*	0.09±0.005
EZB20	0.38±0.025*	0.18±0.011*	0.12±0.009
EZB22	0.31±0.019	0.13±0.010	0.10±0.007

Note: All data are averages ± SE; $n = 30$.
*Significantly different from noninoculated plants ($P < 0.05$).

Plant physiology

Osmotic potential was measured to confirm that PEG 6000 caused osmotic stress (Fig. 1). Medium osmotic potential, Ψ, ranged from –0.15 to –0.23 (unstressed) and –0.26 to –0.35 MPa (stressed) and was significantly affected by the addition of PEG 6000 (Fig. 1). The pH value of the nutrient solutions was not altered by the addition of bacteria or PEG 6000. The humidity in the Magenta boxes was near 100%. Hence, the foliar part of the plants was not exposed to drought stress. Nevertheless, PEG treatment reduced the medium osmotic potential and therefore caused moderate os-

Fig. 2. Content of free proline in leaves of stressed and unstressed pepper plants, either inoculated with strains EZB4 and EZB8 or noninoculated (mean values ± SE; *Significantly different from unstressed control ($P < 0.05$)).



motic stress. The slight decrease of the osmotic potential of the roots of stressed plants is a result of this moderate stress, which was applied for 3 days.

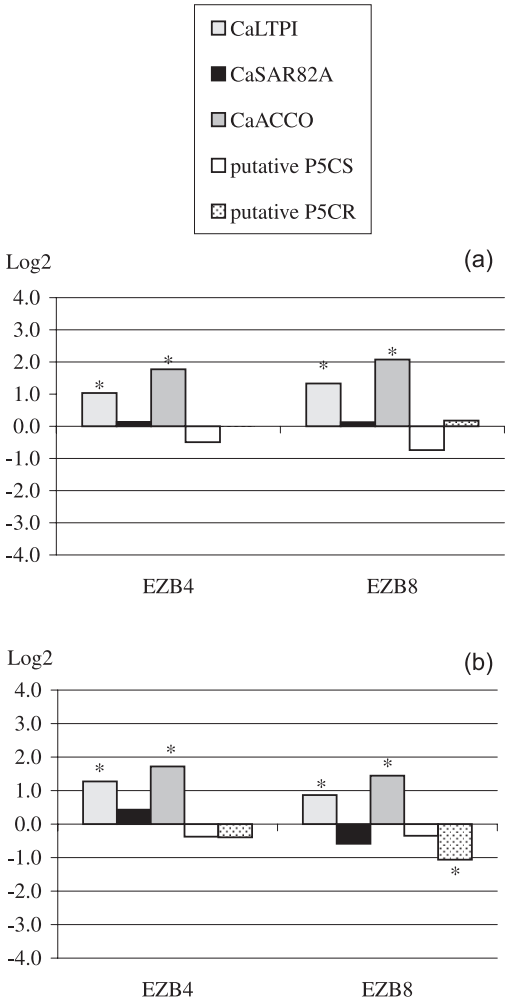
The proline level in leaves of noninoculated plants increased significantly owing to PEG treatment (Fig. 2). Inoculated stressed plants also accumulated the osmolyte proline to nearly the same level. However, the proline concentration was higher in leaves of unstressed plants inoculated with strains EZB4 and EZB8 compared with unstressed noninoculated plants (Fig. 2). Proline is a key metabolite that is synthesized in response to a wide range of biotic and abiotic stresses and mediates osmotic adjustment, stabilizes subcellular structures, and scavenges free radicals (Hare and Cress 1997). Proline accumulation has been reported to improve salt tolerance in transgenic potato plants (Hmida-Sayari et al. 2005). It has also been shown that the plant growth-promoting endophyte *Burkholderia phytofirmans* PsJN increased the levels of free proline, starch, and phenolics in grapevine plantlets, which exhibited an enhanced cold tolerance (Ait Barka et al. 2006). Furthermore, it has been shown in *Arabidopsis thaliana* that proline accumulated in leaf tissue upon recognition of avirulent races of *Pseudomonas syringae* pv. *tomato* (Fabro et al. 2004). Therefore, proline accumulation in unstressed inoculated plants may be the result of biotic stress caused by the endophytic bacteria. It remains to be investigated whether increases in plant proline levels resulting from potential plant–microbe interactions may contribute to better adaption of the plant to stress.

Gene expression in nonstressed plants

Real-time RT–PCR is a sensitive method to evaluate gene expression, provided that the experimental setup allows for good calibration by using genes with steady expression levels as a reference. In this study, polyubiquitin was taken as the endogenous standard instead of actin, as it was revealed to be more stable under the conditions used (data not shown). A reliable internal control gene should show minimal changes in its expression. However, many studies have shown that housekeeping genes can vary with the experimental conditions (Brunner et al. 2004; Nicot et al. 2005).

Inoculation with strains EZB4 and EZB8 altered the gene expression patterns in roots and leaves of unstressed plants in a similar manner (Figs. 3*a* and 3*b*). *CaLTPI* and *CaACCO* were significantly upregulated in roots and leaves to approx-

Fig. 3. Logarithmic scale ratios of gene expression in leaves (*a*) and roots (*b*) of noninoculated vs. inoculated unstressed pepper plants. *Significant ($P < 0.05$).



imately the same extent, whereas no significant changes were observed for *CaSAR82A* and putative proline related *P5CS*. Putative *P5CR* was only significantly downregulated in roots of plants inoculated with strain EZB8.

Recent publications demonstrated via microarray analysis that expression of many plant genes may be altered by beneficial plant-associated bacteria (Cartieaux et al. 2003; Wang et al. 2005). Interestingly, *Pseudomonas fluorescens* FPT9601-T5 caused downregulation of some ethylene-responsive genes, which is in contrast to the upregulation of *CaACCO* attributed to strains EZB4 and EZB8 in our experiment.

CaACCO and *CaLTPI* have been shown to be transcriptionally activated via bacterial and fungal pathogens (Garcia-Pineda and Lozoya-Gloria 1999; Jung et al. 2003), indicating that the strains EZB4 and EZB8 analyzed in this study might have induced at least mild biotic stress. On the other hand, *CaSAR82A* was not differentially expressed, although it is inducible by pathogen infection as well (Lee and Hwang 2003). *CaSAR82A* belongs to a small gene family that is related to systemic acquired resistance. These results suggest that the ethylene pathway is activated by

elicitation of strains EZB4 and EZB8 but not the systemic acquired resistance pathway.

Gene expression in stressed plants

Under moderate osmotic stress conditions, gene expression levels differed significantly between noninoculated and inoculated plants (Figs. 4*a* and 4*b*). *CaACCO* was significantly upregulated in leaves and roots of noninoculated control plants because of the PEG treatment. Inoculation with strain EZB4 resulted in a much lower, yet significant upregulation of *CaACCO* in leaves of stressed plants but was without any effect on the gene expression in roots. No stress effect on *CaACCO* expression was observed in plants inoculated with strain EZB8.

This gene encodes the enzyme ACC oxidase, which catalyzes the final step of ethylene biosynthesis and is known to be strongly induced under stress conditions (Garcia-Pineda and Lozoya-Gloria 1999). Both endophytes showed ACC deaminase activity, which can be found in a wide range of Gram-negative and Gram-positive bacteria, as well as in fungi (Glick 2005). The treatment of plants with ACC-deaminase-producing plant growth-promoting bacteria is an effective means of decreasing ethylene-mediated damage to plants (Glick 2005). Although it might be speculated that ACC deaminase activity is involved in the lower expression of *CaACCO* in comparison with untreated plants, more detailed investigations have to be performed to elucidate the involved mechanisms.

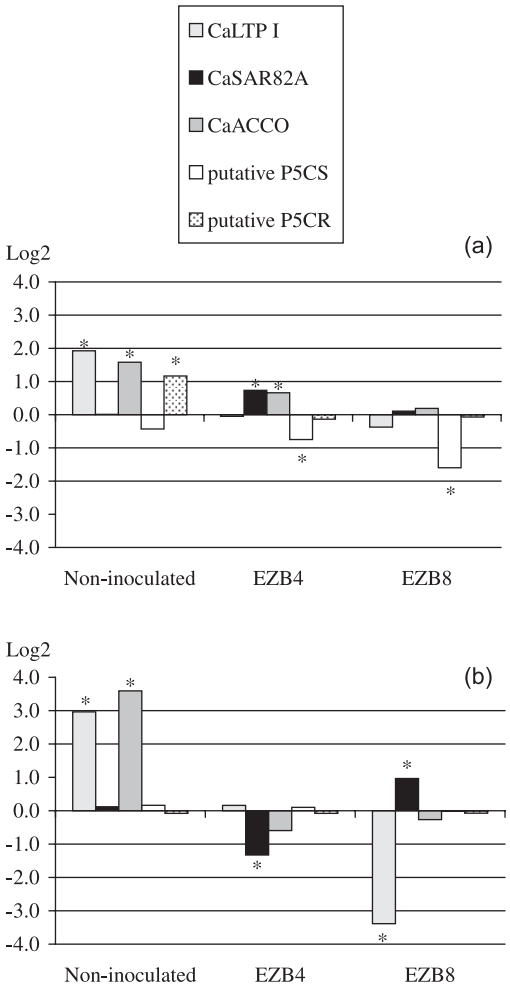
CaLTPI is the second gene that was significantly upregulated in leaves and roots of noninoculated plants because of mild osmotic stress (Figs. 4*a* and 4b). Inoculation with strain EZB4 resulted in a stable gene expression in leaves and roots. In contrast, a significant downregulation was observed in roots inoculated with strain EZB8, but no modulation of gene expression was detected in the leaves.

CaLTPI encodes a lipid transfer protein from pepper and is induced by drought, high salinity, low temperature, and wounding stress, as well as by ethylene, methyl jasmonate, and abscisic acid (Jung et al. 2003). Because ethylene acts as a signalling compound and *CaLTPI* is upregulated by ethylene, a decreased ethylene production caused by the inoculant strains may be the reason for these results.

CaSAR82A is rapidly triggered by high salinity, drought, ethylene, and biotic stress (Lee and Hwang 2003). Under our test conditions, it was differentially expressed in inoculated plants depending on the tissue and strain used, whereas in noninoculated plants, neither in roots nor in leaves was a differential expression found (Figs. 4*a* and 4*b*). Generally, this gene was upregulated under stress conditions, indicating a stress response in the plant that was due to inoculation. However, the lack of effects under unstressed conditions as well as the downregulation of *CaSAR82A* in stressed plants inoculated with strain EZB4 contradict this hypothesis.

Since, in response to osmotic stress, proline is known to accumulate (Hare and Cress 1997), the expression levels of putative *P5CS* and putative *P5CR* were also evaluated. Both genes most probably encode enzymes catalyzing subsequent steps in proline biosynthesis. Under stress conditions in plants, proline is synthesized from glutamate via glutamic-γ-semialdehyde, catalyzed by P5CS, and subsequent reduction of Δ^1-pyrroline-5-carboxylate by P5CR (Mazzola et al. 2004; Miché et al. 2006).

Fig. 4. Logarithmic scale ratios of gene expression in leaves (*a*) and roots (*b*) of stressed vs. unstressed pepper plants inoculated and noninoculated with endophytes. *Significant ($P < 0.05$).



Genes tested were a putative *P5CS* showing 87.97% similarity to tomato *P5CS* and a putative *P5CR* belonging to a *P5CR*-related cluster. Stress did not affect the expression of these genes in roots, whereas a clear downregulation due to bacterial inoculation under stress conditions was determined in leaves (Fig. 4*a*). In contrast, *P5CR* was significantly upregulated in noninoculated plants but not differentially expressed in plants inoculated with endophytes. This is in complete contrast to results reported of *Arabidopsis thaliana* under osmotic stress, where the *P5CS* gene was inducible by drought stress, salinity, and abscisic acid, but *P5CR* was not (Yoshiba et al. 1995).

Regarding the results obtained with inoculated plants, a similar effect was observed in soybean and lettuce (Porcel et al. 2004). Plants inoculated with *Bradyrhizobium japonicum* also showed lower *P5CS* transcript accumulation under drought stress than in noninoculated plants (Porcel et al. 2004). Both endophytes tested in this study caused higher proline contents in leaves of plants not treated with PEG. Although these amounts were not significant because of large variations, they may be the reason for the significant

downregulation of putative *P5CS* gene in leaves, considering the fact that the P5CS protein and, probably *P5CS* gene expression, is feedback inhibited by proline (Porcel et al. 2004).

In conclusion, our results indicated that the 2 endophytic strains investigated in more detail exposed mild biotic stress to the pepper plant. Nevertheless, these strains increased plant biomass after 3 weeks and induced abiotic stress relief. It must be considered that this experiment was made in closed Magenta boxes and that the stress applied was moderate. However, it is likely that under natural conditions, plant-associated bacteria exhibit the same or similar effects in cases where they colonize plants efficiently. Our results indicate that endophytes play an important role in plant stress tolerance and may find application to enhance abiotic stress resistance.

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