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DISSERTATION

Titel der Dissertation

„Effects of Early Drought Stress and Bacterial Endophytes on Gene Expression and Plant Physiology in Pepper (*Capsicum annuum* L.)"

Verfasserin

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(Pictures by C. Naglreiter and W. Sziderics)

Different varieties of *C. annuum* (1st and 2nd row) and *C. chinense* (3rd row)

1st Row: Milder Spiral, Lozorno, Abbraccio

2nd Row: Kalinko, Ziegenhorn Bello, Tequilla

3rd Row: Habanero, Fatallii, Bhut Jolokia

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ABSTRACT

Water deficit is one of the major limitations in pepper production. A fundamental understanding of the physiological and molecular networks is essential to develop drought tolerant plants. Furthermore endophytic bacteria can enhance stress resistance. In this thesis early drought stress response in different plant organs as well as the effect of endophytic bacteria on physiological parameters and gene expression were analysed. Biochemical measurements, real-time RT-PCR and suppression subtractive hybridisation (SSH) were coupled with microarray analyses. As for pepper no microarray platform exists probes of putative drought responsive genes of different plant species were added to the microarray resulting in a stress-specific multi-species array.

Pepper plants adapted to water deficit by adjustment of their osmotic potential and accumulated raffinose and glucose in roots. In contrast amounts of fructose and sucrose increased in leaves. Galactinol and hydroxyproline levels were elevated in both plant organs. In leaves diverse polyamines were accumulated too, whereas their content was reduced in roots. The content of proline increased tremendously in roots but was reduced in leaves. Increased levels of proline were also found in plants inoculated with endophytic bacteria. Gene expression of ESTs encoding for enzymes related to proline metabolism matched the metabolic profile of the stressed plants. Inoculation with endophytes resulted in changed expressions of two proline synthesis related genes. The 1-aminocyclopropane-1-carboxylate (ACC) deaminase producing bacteria also altered the expression of an EST encoding for ACC oxidase, which catalyses the final step of ethylene biosynthesis.

Microarray analyses of the clones of the SSH-libraries resulted in 109 unique drought responsive ESTs. Their expression differed within leaves and roots, but none was regulated into opposite direction within the two organs. In addition 286 ESTs were related to forward or reverse libraries by hybridisation of the array with subtracted cDNAs. The sequenced ESTs could be assigned to a broad spectrum of functional classes, which reflects the complexity of the response of the plants. Hybridisation of the multi-species microarray with cDNAs of stressed pepper plants resulted in hybridisation rates ranging from 57.9% to 95.7% depending on the plant species and their phylogenetic distance to pepper. Analysis of homologous genes revealed a distinct loss of power. However, none of the analysed expression rate of the cross-species probes was in clear contrast to the performance of the pepper probes.

In summary this thesis provides new insights into drought stress response of pepper plants and suggests abiotic stress reduction by bacterial endophytes.

KURZFASSUNG

Wassermangel ist einer der Hauptfaktoren, die die Paprikaproduktion beschränken. Grundlegendes Wissen über die physiologischen und molekularbiologischen Zusammenhänge sind Voraussetzung für die Entwicklung trockenheitstoleranter Pflanzen. Auch durch Endophyten kann die Stresstoleranz gesteigert werden. In dieser Arbeit wurden die Reaktionen bezüglich physiologischer Parameter und der Genexpression in verschiedenen Pflanzenorganen von Paprika auf beginnenden Trockenstress sowie die Wirkung endophytischer Bakterien untersucht. Biochemische Analysen, real-time RT-PCR und Suppression Subtractive Hybridisation wurden mit Microarray-Analysen verbunden. Da es für Paprika keine repräsentative Microarray-Plattform gibt, wurden vorhandene, mutmaßlich auf Trockenstress reagierende ESTs verschiedener Pflanzenspezies, zusätzlich auf das Microarray gespottet, wodurch ein stress-spezifisches Mulit-Species-Array entstand.

Die Pflanzen reagierten auf den Wassermangel mit Anpassung des osmotischen Potentials und Anreicherung von Raffinose und Glucose in den Wurzeln. In den Blättern nahm dagegen der Gehalt an Fructose und Saccharose zu. Die Werte für Galactinol und Hydroxyprolin stiegen in beiden Pflanzenorganen an. In den Blättern reicherten sich außerdem verschiedene Polyamine an, deren Gehalt in den Wurzeln reduziert war. Im Gegensatz dazu nahm der Prolin-Gehalt in den Wurzeln enorm zu und in den Blättern ab. Erhöhte Mengen Prolin wurden auch in den Pflanzen, die mit den Endophyten inokuliert wurden, festgestellt. Die Genexpressionen von ESTs, die Enzyme kodieren, die im Zusammenhang mit dem Stoffwechsel von Prolin stehen, paßten mit dem metabolischen Profil der Pflanzen überein. Die Inokulation mit Endophyten führte zu einer Veränderung der Regulierung von zwei Genen, die mit der Prolin-Synthese in Zusammenhang stehen. Durch die 1-Aminocyclopropan-1-Carboxylate(ACC)-Deaminase produzierenden Bakterien änderte sich auch die Expression des Gens, welches das Enzym ACC Oxidase codiert. Dieses Enzym katalysiert den letzten Schritt der Ethylen-Synthese.

Eine Microarray-Analyse der Klone der SSH-Libraries ergab 109 trockenstress-induzierte ESTs. Zwar wurden diese in Blättern und Wurzeln unterschiedlich reguliert, jedoch nicht in gegensätzliche Richtungen. Durch Hybridisierung des Microarrays mit subtrahierter cDNA konnten zusätzlich 286 ESTs einem Forward- oder Reverse-Library zugeordnet werden. Das breite Spektrum der funktionellen Klassen der sequenzierten ESTs spiegelt die Komplexität der Stressantwort der Pflanzen wider.

Die Hybridisierung des Multi-Species-Microarrays mit cDNA gestresster

Paprikapflanzen resultierte in Abhängigkeit von der Pflanzenart und derer phylogenetischen Distanz zu Paprika in Hybridisierungsraten zwischen 57,9% und 95,7%. Zwar ergab die Analyse homologer Gene einen deutlichen Verlust an Aussagekraft durch die Verwendung verschiedener Spezies, jedoch standen die mit den Sonden der verschiedenen Spezies gemessenen Genexpressionen nicht im deutlichen Gegensatz zur Performance der Paprika-Sonden.

Zusammenfassend zeigt diese Arbeit neue Einblicke in die Auswirkungen von beginnendem Trockenstress auf Paprikapflanzen und gibt einen Hinweis auf eine mögliche Stressreduktion durch bakterielle Endophyten.

1. INTRODUCTION

1.1. Pepper (Capsicum annuum L.)

The genus *Capsicum* belongs to the *Solanaceae* family and is endemic to the western hemisphere. Its natural range extended from the southern border of the United States to the temperate zone of southern South America (Heiser, 1976). *Capsicum* probably evolved from an ancestral form in the Bolivian/Peru area (Heiser, 1976). It consists of approximately 22 wild species and five domesticated species (Bosland, 1994): *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens*. Chili peppers have been shown to be domesticated more than 6000 years ago (Perry et al., 2007). After Christoph Columbus reached America several pepper species were cultivated in Spain and are nowadays produced world wide. The most economically important species in the world is *C. annuum* (Greenleaf, 1986; Bosland et al., 1988).

In Austria pepper production increased continuously over the last 10 years from 125 ha (1998) to 157 ha (2008). The production shifted from capia pepper used for processing (930 t in 2008) to coloured and green bell pepper used as fresh fruits (16500 t in 2008). Nearly 75% of the pepper in Austria is produced under protected cultivation (Statistik Austria, 1998; Statistik Austria, 2008).

Pepper is a warm-season crop and sensitive to freezing temperatures at all growth stages. For germination it requires relatively high temperatures with an optimum temperature around 25°C – 30°C (Sachs et al., 1980; O'Sullivan and Bouw, 1984). At lower temperatures germination is delayed. Capsicums flourish in warm sunny conditions, and require 3-5 months with a temperature range of 18°C - 30°C. At temperatures below 5°C growth is retarded (Douglas et al., 2005). Flowers are sensitive to high temperatures. Exposure of flowers buds to 33 °C for two days resulted in reduced fruit set and impaired pollen viability. Also high post-pollination temperatures inhibit fruit set (Erickson and Markhart, 2002).

Pepper is considered as one of the most sensitive crops to soil water deficit (González-Dugo et al. 2007). For high yields an adequate water supply and moist soils are required during the total growing period. Production of pepper in aride and semiaride regions as well as pepper grown under protected cultivation require irrigation. Drip irrigation is very effective and supplies water and nutrients at a rate that is close to plant uptake. However, in many production regions irrigation systems are not feasible due to water deficit or high salinity. Reduction in water supply during the growing period in general has an adverse effect on yield. Low water availability prior and during flowering reduces the number of flowers

and fruits (Jaimez et al., 2000). Thus water deficit is one of the major factors limiting pepper productivity.

1.2. Drought stress

Drought is one of the most wide spread environmental stresses reducing yields by as much as 50% (Bray et al., 2000). According to the assessment report of the Intergovernmental Panel on Climate Change (IPCC, 2007) drought-affected areas are expected to increase with the potential for adverse impacts on multiple sectors, e.g. agriculture, water supply, energy production and health. Consequently, the development of drought-tolerant varieties will become increasingly important and efforts are directed towards a better understanding of plant responses to water deficit.

Plant adaptation to environmental stresses is controlled by cascades of molecular networks resulting in a combination of metabolic, physiological and morphological changes (Fig. 1). Stress perception by osmosensors leads to signal transduction via primary and secondary messengers. Secondary signals can be phytohormones such as abscisic acid (ABA) and ethylene as well as Ca^{2+}, reactive oxygen species and intracellular second messengers such as phospholipids (Xiong and Zhu, 2002). Controlled by regulatory proteins such as transcription factors, mitogen activated and calcium-dependent protein kinases and phospholipases, stress associated genes encoding functional proteins or again regulatory proteins are activated (Shinozaki and Dennis, 2003; Beck et al., 2007). Thus enzymes involved in stress avoidance by maintaining the osmotic pressure and stabilisation of the quaternary structure of proteins as well as proteins involved in damage repair are synthesised.

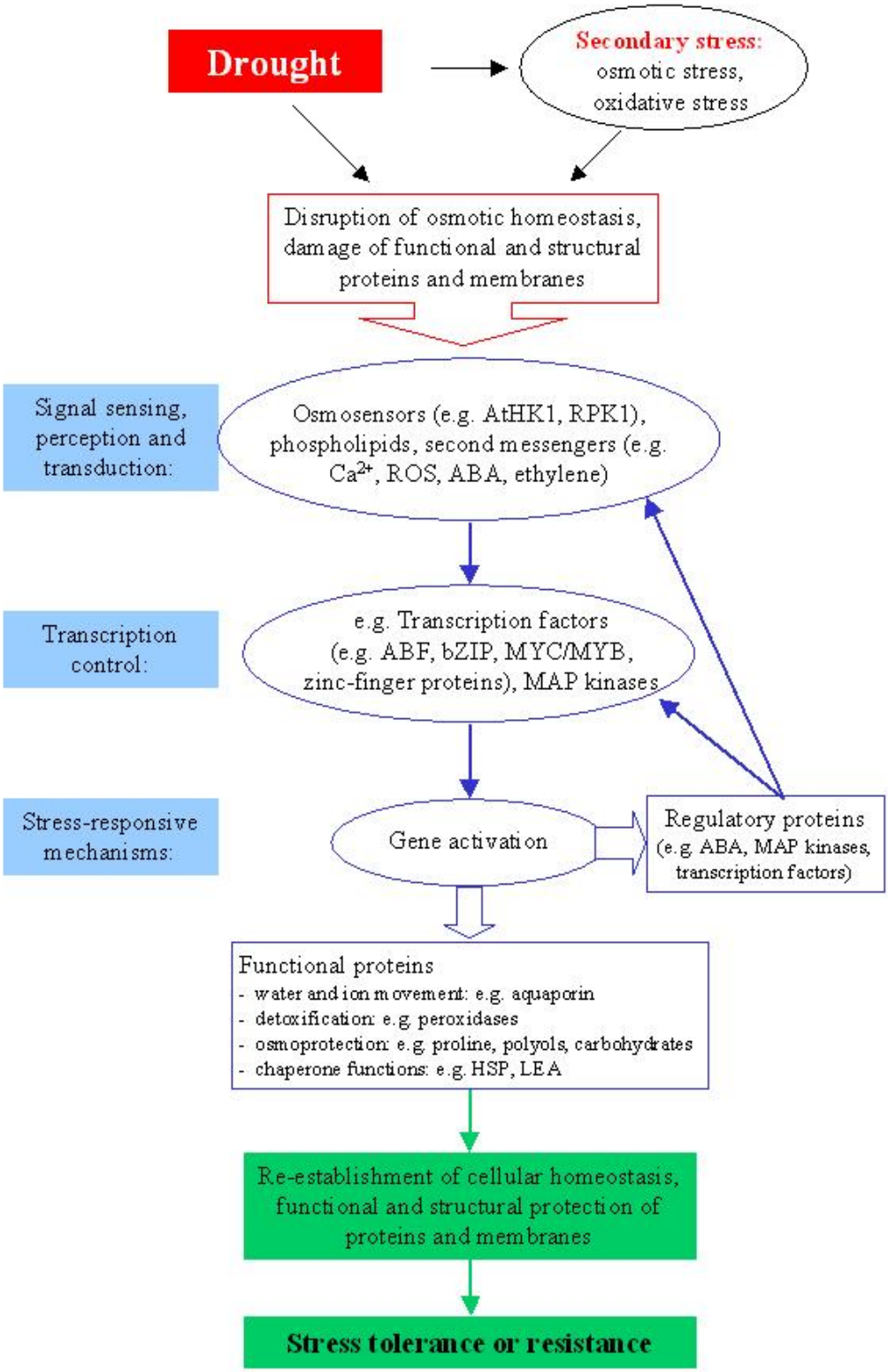

Fig 1: Plant responses to drought stress (adapted from Vinocur and Altman, 2005 and Beck et al., 2007)

1.2.1 Stress avoidance and damage repair

One prominent response to drought stress is the modulation of the osmotic level of the plant cell's cytosol and the vacuoles by accumulation of manifold substances, with the aim to counteract the loss of turgor (Cushman, 2001). These osmolytes do not interfere with normal cellular biochemical reactions, but help to maintain an osmotic balance. Osmolytes include amino acids, such as proline and quaternary ammonium compounds (e.g. glycine betaine), hydrophilic proteins (e.g. late embryogenesis abundant proteins), carbohydrates (e.g. sucrose, trehalose, fructan), polyols (e.g. pinitol, mannitol) and polyamines (Chaves et al., 2003; Liu et al., 2004). Accumulation of proline and glycine betaine under water deficit and salt stress have been demonstrated in many different plants (Delauney and Verma, 1993; Rhodes and Hanson, 1993). Interestingly several plant species such as *Arabidopsis* and tobacco do not synthesise glycine betaine (Sakamoto and Murata, 2000). Increased levels of sucrose and/or reducing sugars have been also frequently reported and proposed to contribute towards the maintenance of turgor (Hare et al., 1998). For example high concentrations of the monosaccharides glucose and fructose were measured in maize and soybean leaves upon osmotic stress (Pellechi et al., 1997; Liu et al., 2003). Also sugar alcohols and cyclic polyols are known to accumulate under drought stress. Mannitol, a six carbon polyol, is the most widely distributed sugar alcohol in nature (Stoop et al., 1996) and has been shown to accumulate under drought stress for example in *Fraxinus* (Guicherd P. et al., 1997). Increased concentrations of the cyclic pinitol were demonstrated in white clover (McManus et al., 2000) and soybean (Streeter et al., 2001).

Beside their contribution to osmotic adjustment some solutes may also function as osmoprotectants for example in radical oxygen scavenging or stabilisation of proteins, protein complexes or membranes at low, osmotically insignificant concentrations (Bohnert and Shen, 1999).

1.2.1.1 Proline

One of the best studied compatible solute is the amino acid proline. In plants proline is synthesised from glutamate via Δ1-pyrroline-5-carboxylate (P5C) by two successive reductions catalysed by P5C synthetase and P5C reductase. Genes encoding these enzymes have been identified in several plant species and have been shown to be up-regulated under osmotic stress (Hare et al., 1999). In addition in higher plants proline can be synthesised from ornithine by transamination to alpha-keto-delta-aminovalerate which is in spontaneous equilibrium with pyrroline-2-carboxylate (P2C), which can be reduced to proline. Although

there is also the possibility of delta-NH_2 transamination resulting in glutamic-γ–semialdehyde which is in spontaneous equilibrium with P5C, the P2C pathway of ornithine metabolism is suggested to be the main alternative route of conversion of ornithine to proline (Mestichelli et al., 1979). Degradation results from oxidation of proline to P5C by proline dehydrogenase and subsequent formation of L-glutamate by P5C dehydrogenase.

Under osmotic stress proline metabolism is increased, whereas its catabolism is repressed (Kavi Kishor et al., 2005). The accumulation appears to be mediated by both ABA-dependent and ABA-independent signalling pathways (Hare et al., 1999). Beside its role as compatible solute proline is suggested to also reduce protein denaturation and thus preserve enzyme structures and activities (Samuel et al., 2000) and may serve as a source of carbon, nitrogen and energy during recovery from stress (Hamilton and Heckathorn, 2001).

1.2.1.2 Glycine betaine

Glycine betaine is a quaternary ammonium compound, which is synthesised in most plants from choline in two oxidation steps via the intermediate betaine aldehyde. The relevant enzymes isolated from several higher plants are a ferredoxin-dependent choline monooxygenase and betaine aldehyde dehydrogenase (Sakamoto and Murata, 2000). The immediate product of the choline monooxygenase reaction may by betaine aldehyde hydrate, which is in spontaneous equilibrium with betaine aldehyde (Lerma et al., 1988). Corresponding genes encoding choline monooxygenase and betaine aldehyde dehydrogenase have been isolated from spinach and sugar beet (McCue and Hanson, 1992; Rathinasabapathi et al., 1997; Weretilnyk and Hanson, 1990). However, it must be considered that betaine aldehyde dehydrogenase may act also on other aldehyde substrates (Trossat et al., 1997). This multiple substrate specificity may explain its occurrence in plants that do not accumulate glycine betaine such as rice, tobacco or potato (Ishitani et al., 1993; Weretilnyk and Hanson, 1989; McCue and Hanson, 1990; Holmström et al., 2000).

Under abiotic stress glycine betaine is not only an effective compatible solute acting as osmoregulator, but also stabilises the structures and activities of enzymes and protein complexes and maintains the integrity of membranes against effects of excessive salt, cold and heat (Sakamoto and Murata, 2002). It is also suggested to stabilise RuBisCo conformation under high salt conditions and to play a role in the repair of the PSII complex during photoinhibition (Sakamoto and Murata, 2002). It is not effective against reactive oxygen species as a hydroxyl radical scavenger (Smirnoff and Cumbes, 1989).

1.2.1.3 Carbohydrates

Carbohydrates play a central role in plant metabolism and hence regulate plant growth and development. Sucrose and its derivatives are the major transport forms of assimilated carbon in plants. For long distances in most plants sucrose is transported from source to sink organs via the phloem. Transmembrane transport of sucrose and other solutes requires transporters such as the phloem associated sucrose transporter SUT1, which is known to be essential for sucrose translocation in potato and tobacco (Lalonde et al., 1999). Genes encoding sucrose transporters as well as monosaccharide transporters have been identified in several plant species. The transport of sugars is of importance as sugar acts as signalling substance in plants. Many genes involved in photosynthesis, carbon and nitrogen metabolism and stress response including ABA-inducible genes are regulated at the transcriptional level by sugars (Rolland et al., 2002). It has also been shown, that accumulation of soluble sugars negatively regulate photosynthesis gene expression including expression of Calvin cycle genes (Couée et al., 2006).

1.2.1.4 Polyols

Polyols are low molecular weight, highly soluble, reduced forms of aldose and ketose sugars. In plants frequently found linear polyols are mannitol and sorbitol and the cyclic forms galactinol, *myo*-inositol and pinitol. Mannitol and sorbitol are direct photosynthetic products in parallel with sucrose and have similar functions in translocation of assimilated carbon (Lewis, 1984; Loescher, 1987). *Myo*-inositol is synthesised from of D-glucose-6-phosphate and is a central component of further biochemical pathways. It can be converted into pinitol via ononitol by methylation and epimerisation (Dittrich and Brandl, 1987). Further *myo*-inositol may be converted into galactinol by addition of UDP-galatose catalysed by galactinol synthase. As raffinose is formed from galactinol and sucrose, galactinol synthase catalyses the first step in the biosynthesis of the raffinose family oligosaccharides and thus plays a key regulatory role in carbon partitioning between sucrose and raffinose oligosaccharides.

Upon drought stress polyols increase in several plant species and are suggested to have an osmotic effect (Guicherd et al., 1997; McManus et al. 2000; Streeter et al. 2001; Taji et al., 2002). Galactinol has also been shown to effectively protect salicylate from hydroxyl radicals in vitro. Thus galactinol may also act as scavenger of hydroxyl radicals and protect plant cells from oxidative damage (Nishizawa et al., 2008). Mannitol has also been shown to act as hydroxyl radical scavenger in vitro and in vivo (Smirnoff and Cumbes, 1989; Shen et

al., 1997a). Phosphoribulokinase seems to be one of the target enzymes protected by mannitol (Shen et al. 1997b).

1.2.1.5 Polyamines

Polyamines are small organic compounds with two or more primary amino groups found in all eukaryotic cells. Putrescine, spermidine and spermine are the major polyamines found in plants involved in various processes such as cell proliferation, growth, morphogenesis, differentiation and programmed cell death (Yamaguchi et al., 2007).

In plants putrescine is either synthesised directly from ornithine by ornithine decarboxylase (ODC) or from arginine via *N*-carbamoylputrescine and agmatine. Conversion of arginine requires the enzymes arginine decarboxylase (ADC), *N*-carbamoylputrescine amidohydrolase and agmatine deiminase (Urano et al., 2003). Putrescine can be further converted into spermidine and consequently to spermine by spermidine or spermine synthases (SPDS, SPMS) by addition of an aminopropyl moiety from decarboxylated S-adenosylmethionine generated by S-adenosylmethionine decarboxylase (SAMDC). S-adenosylmethionine is also the precursor of aminocyclopropane carboxylic acid, an important source of ethylene, thus polyamine and ethylene metabolism are linked together. The less common polyamine cadaverine is synthesised by a direct decarboxylation of lysine (Bakhanashvili et al., 1985).

In *Arabidopsis* genes involved in polyamine synthesis were identified encoding ADC, SAMDC, SPDS and SPMS (Urano et al. 2003), *N*-carbamoylputrescine amidohydrolase (Piotrowski et al., 2003) and agmatine iminohydrolase (Janowitz et al., 2003). Under drought stress *AtADC2* and *AtSPMS* were clearly induced (Urano et al., 2003).

Accumulation of putrescine, spermidine, spermine and cadaverine is well known under drought stress and has been reported in many plant species. Beside their possible effect on the osmotic adjustment polyamines are also involved in stomata closure by regulation of the voltage-dependent inward K^+ channel in the plasma membrane of guard cells (Liu et al., 2000). All natural polyamines have been shown to strongly inhibit opening and to induced closure of stomata. In addition polyamines are known to be components of the cellular antioxidant system and are usually regarded as scavengers of hydroxyl radicals. Kuznetsov et al. (2007) demonstrated *in vitro* that cadaverine inhibited DNA oxidative degradation by the hydroxyl radical-generating system. Also putrescine, spermidine and spermine were found to be potent scavengers of hydroxyl radicals in a dose dependant manner. In addition spermine or spermidine were shown to quench singlet oxygen at higher concentrations(Das and Misra,

2004). However, at 0.5, 1.0 and 2 mM concentrations neither cadaverine nor putrescine, spermidine or spermine could scavenge superoxide radicals (Das and Misra, 2004).

1.2.1.6 Late embryogenesis abundant proteins

Late embryogenesis abundant proteins (LEA) are a major group of proteins that typically accumulate during late stages of embryogenesis or in response to dehydration, low temperature salinity or exogenous ABA treatment – indicating their responsiveness to cellular dehydration. These proteins have a biased amino acid composition, and are typically highly water soluble and hydrophilic (Ramanjulu and Bartels, 2002). The most frequently induced group of LEA proteins under water deficit are dehydrins. However, some atypically hydrophobic LEA proteins were also identified such as for example *CaLEA6* (Kim et al., 2005). In *Arabidopsis* Hundertmark and Hincha (2008) identified 51 LEA protein encoding genes. Most of these genes had ABA response (ABRE) and/or low temperature response (LTRE) elements in their promoters and many genes containing the respective promoter elements were induced by ABA, cold or drought. In connection with drought LEA proteins were proposed to have functions like binding or replacement of water, sequestration of ions, macromolecule and membrane stabilisation (Close, 1996; Heyen et al., 2002). Several proteins could be shown to have a high hydration capacity and to bind a large amount of charged solute ions (Tompa et al., 2006), to prevent protein aggregation (Goyal et al., 2005), to interact with membranes and to protect liposomes (Tolleter et al., 2007) or to bind to lipid vesicles (Koag et al., 2003) or actin filaments (Abu-Abied et al., 2006).

1.2.1.7 Heat shock proteins

Heat shock proteins (HSPs) form a big protein family including proteins of different physiological functions and are known to be involved in plant abiotic stress response. HSPs function as intra-cellular chaperones for other proteins. They play an important role in protein folding, stabilising partially unfolded proteins and prevention of unwanted protein aggregation (Borges et al., 2005). High temperatures as well as salinity and drought stress can cause denaturation and dysfunction of proteins. It is suspected, that HSPs protect plants by controlling the proper folding and conformation of structural proteins like membranes and functional proteins (Vanocur and Altman, 2005).

Transcription of genes encoding heat shock proteins is primarily regulated at the transcriptional level by heat stress transcription factors, which are activated by stress that leads to a specific binding to the heat shock promoter element (Wu, 1995; Nover et al. 1996).

Genes encoding HSPs and heat stress transcription factors have been frequently reported to be up-regulated under drought stress (Seki et al., 2002; Kawaguchi et al., 2004; Busch et al., 2005; Swindell et al., 2007).

1.2.1.8 Reactive oxygen species scavengers

Drought stress decreases CO_2 assimilation rate due to reduced stomatal conductance. The capacity of the electron transport chain in such conditions exceeds the consumption of reduction equivalents delivered to the stroma side of the thylakoid membranes. Duration of this constraint is harmful to plants, because it triggers the production of reactive oxygen species (ROS), such as hydroxyl radicals, singlet oxygen, superoxide and hydroxy peroxide (Kotchoni and Bartels, 2003). These molecules generated within the chloroplasts can damage the photosynthetic apparatus. Antioxidant molecules and enzymes located in different cell compartments can scavenge ROS. These include superoxide dismutases (SODs), which catalyse the dismutation of $O_2^{\bullet-}$ to H_2O_2, catalases and enzymes and metabolites of the ascorbate-glutathion cycle that are involved in the removal of H_2O_2 (Chaves M.M. et al., 2003). The balance between SODs and the different H_2O_2-scavenging enzymes in cells is considered to be crucial in determining the steady-state level of $O_2^{\bullet-}$ and H_2O_2 (Mittler et al., 2004). Under drought stress genes encoding scavenging enzymes are known to be up-regulated (Reddy et al., 2004).

1.2.2 Signaling

A generic signal transduction pathway starts with signal perception by for example receptor-like kinases followed by the generation of second messengers and activation of stress responsive genes, whereas second messengers such as plant hormones and reactive oxygen species can initiate another series of signaling events. The activation of stress responsive genes is further modulated by phosphoprotein cascades and transcription factors (Shinozaki and Dennis, 2003; Xiong et al., 2002). The manifold features of gene families encoding signaling molecules, protein kinases and transcription factors provide complexity and flexibility in plants responses to environmental stresses.

An universal secondary messenger responding to stress is Calcium. Changes in cytosolic Ca^{2+} levels can be sensed by calcium-dependent protein kinases (CDPKs), which modify the phosphorylation status of substrate protein (Knight and Knight, 2001). Several genes encoding CDPKs of broad bean (*Vicia faba)* and wheat (*Triticum aestivum* L.) have been shown to be induced under drought stress (Liu et al., 2006; Li et al., 2008). Besides

CDPKs mitogen activated protein kinases (MAPKs) play an important role in cell signaling. MAPK cascades amplify and transmit signals through a series of phosphorylation events from MAPKKK to MAPKK to MAPK (Cardinale et al., 2002). Targets of MAPKs can be again protein kinases as well as various transcription factors. It has further been shown, that MAPK signaling pathways can regulate ROS production (Pitzschke and Hirt, 2009). Beside their negative effect on cell damage ROS can also act as signalling molecules as they are small and able to diffuse over a short distance. Among different ROS only H_2O_2 can cross plant membranes and therefore can directly function in cell-to-cell signaling (Pitzschke and Hirt, 2006). Furthermore H_2O_2 has been shown to change the intercellular calcium levels to mediate stomatal closure (McAinsh et al, 1996). Downstream signaling events associated with ROS sensing involve Ca^{2+} and Ca^{2+}-binding proteins, the accumulation of phospholipid signaling and mitogen-activated protein kinase (MAPK) cascades (Mittler et al., 2004). In Arabidopsis H_2O_2 has been shown to activate the MAPKKK ANP1, which further initiates phosphorylation cascade involving two stress MAPKs, AtMPK3 and AtMPK6 (Kovtun et al., 2000).

After signal sensing, perception and transduction gene expression is controlled by transcription factors. Among stress inducible transcription factors members of the dehydration-responsive element-binding (DREB) protein family, the ethylene-responsive element binding factor (ERF) family, the zinc-finger family, the WRKY and MYB family, the basic helix-loop-helix family, the basic-domain leucin zipper (bZIP) family, the NAC family and the homeodomain transcription factor family have been identified (Shinozaki et al., 2003). Transcription factors of the bZIP family are known to recognise the core sequence of the ABA-responsive element (ABRE) (Hattori et al., 1995; Choi et al., 2000; Uno et al., 2000). This element has been identified from the promoter analysis of ABA-regulated genes and is described in several plant species. Like ABRE-binding proteins the MYC and MYB transcription factors *AtMYC2* and *AtMYB2* from *Arabidopsis* are known to bind *cis*-elements and suggested to be also involved in the ABA-dependent signaling pathway (Shinozaki et al., 2003). In addition an ABA-independent signalling pathway is known via the dehydration-responsive element (DRE)/C-repeat (CRT). Transcription factors belonging to the ERF/APETALA2 (AP2) family that bind to DRE/CRT have been isolated from *Arabidopsis thaliana* (Stockinger et al., 1997; Liu et al., 1998). NAC transcription factors are suggested to be involved in both the ABA-dependent and ABA-independent pathway (Shinozaki and Yamaguchi-Shinozaki, 2006).

1.2.2.1 Abscisic acid

The natural plant hormone abscisic acid is a sesquiterpenoid derived from C_{40} carotenoids. The S-enantiomer is the natural and active form found in all green plants, also in some mosses, algae and fungi. Upon drought stress the endogenous levels of ABA in leaves can rise more than 10-fold and returns to pre-stress levels once the plants are watered again (Xiong et al. 2002). ABA is synthesised mainly from zeaxanthin. The first step is the conversion of zeaxanthin into all-trans-violaxanthin via antheraxanthin by zeaxanthin epoxydase (ZEP) followed by a conversion into 9-cis-violaxanthin or 9´-cis-neoxanthin. Afterwards 9-cis-epoxycarotenoid dioxygenase (NCED) catalyses the oxidative cleavage of both intermediates into xanthoxin. Xanthoxin can be converted into ABA via xanthoxic acid, abscisic aldehyde or abscisic alcohol, which is formed from abscisic aldehyde (Seo and Koshiba, 2002). Enzymes catalysing these steps are a short-chain dehydrogenase/reductase and aldehyde oxidases. Several genes encoding the catalysing enzymes have been identified. Expression studies in different plant species indicated that drought stress-induced ABA levels correlated well with transcript and protein accumulation of NCED. Thus NCED is suggested to be the initial rate-limiting step in ABA biosynthesis (Iuchi et al. 2001; Xiong et al. 2002). ABA by itself induces the expression of several ABA biosynthesis genes such as *ABA1, ABA3, NCED3* and *AAO3* (León and Sheen, 2003). This is not the case for *ABA2*, which is up-regulated by glucose but not by ABA (Cheng et al., 2002). By contrast, the *NCED3* gene, which is strongly activated by drought and osmotic stress in various plants, appears to be insensitive to glucose in Arabidopsis (Cheng et al., 2002; Iuchi et al., 2000; Qin and Zeevaart, 1999). This suggests that stress- and glucose- induced ABA accumulation is mediated by distinct mechanisms.

ABA has long been known to be important for drought tolerance by mediating guard cell signalling and stomatal closure to prevent extensive water loss. Moreover, it is known to control the expression of many drought stress related genes. Thus under drought stress signalling pathways may be ABA-dependent and ABA-independent. Many ABA-dependent stress-inducible genes contain a motif known as the ABA-responsive element (ABRE) (Zhang et al., 2006), which are target binding sites for b-zip transcription factors (Shinozaki and Yamaguchi-Shinozaki, 2000). In addition expression of genes may be controlled by ABA-inducible MYC and MYB transcription factors (Riera et al., 2005).

1.2.2.2 Ethylene

Besides its physiological roles in different developmental stages the plant hormone

ethylene is also regarded as stress hormone as its synthesis is induced by a variety of stress signals such as drought, extreme temperatures, mechanical wounding or pathogen infection. The gaseous hormone is synthesised from methionine via S-adenosylmethionine (S-AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC). S-AdoMet is also the methyl donor for many cellular molecules including nucleic acids, proteins and lipids and the precursor of the polyamine synthesis pathway. The enzymes catalysing the ethylene biosynthesis steps are SAM synthase, ACC synthase and ACC oxidase (Kende, 1993). The key enzymes ACC synthase and ACC oxidase are both encoded by multi-gene families and have been identified in many different plant species (Kende, 1993; Momonoi et al. 2007).

Ethylene acts as a signalling molecule and triggers genome-wide changes in gene expression. In Arabidopsis five ethylene receptors have been identified. It is assumed, that hormone binding to the receptors results in receptor inactivation (Hua and Meyerowitz, 1998). In absence of ethylene, therefore, the receptors are hypothesised to be in a functionally active form that constitutively activates Raf-like serine/threonine kinase (CTR1) (Guo and Ecker, 2004). Upon ethylene binding the receptors/CTR1 signalling complexes become inactive and thus releasing the repression of the downstream signaling pathway (Benavente and Alonso, 2006). Downstream components in the ethylene pathway include several regulators and ethylene responsive element binding protein (EREBP) transcription factors, which can bind to the promoter of EREBP genes (Guo and Ecker, 2004). Beside modulation of gene expression ethylene is also known to modulate other plant hormones. Tanaka et al. (2005) showed, that ethylene inhibits ABA-induced stomatal closure in Arabidopsis by inhibiting the ABA signalling pathway. Furthermore Rosado et al. (2006) demonstrated extensive cross-talk between ABA and ethylene signalling pathways in tomato. However, the cross point of interaction between ABA and ethylene signalling pathways is not clear.

1.2.3 Gene expression

Drought stress leads to drastic alterations in the gene expression profile. Microarray technology is a powerful tool to analyse expression changes of a large number of genes simultaneously and thus provides new insights into physiological and biochemical pathways. There are two predominant varieties of microarray technology available, the cDNA microarray and the oligonucleotide microarray, the most prominent being the Affymetrix GeneChip. Seki et al. (2002) used a 7000 full-length cDNA microarray to identify 299 drought-inducible genes of *A. thaliana* whereas Kreps et al. (2002) used the Affymetrix GeneChip array containing oligonucleotides representing approximately 8100 independent

Arabidopsis genes. One of the advantages of oligonucleotide array is the design of the probes, which represent unique gene sequences. Depending on the completeness of the sequence information this minimises cross-hybridisation between genes belonging to a gene family or genes with common functional domains. In addition the oligonucleotides are typically designed to have uniform lengths and melting temperatures and are spotted in uniform concentrations. In contrast cDNA libraries can be prepared directly from existing cDNA libraries (Alba et al., 2004). Thus for plant species where currently no representative microarray platform is available cDNA microarrays may be prepared from cDNA libraries. Also subtracted cDNA libraries may be used for microarray preparation and subsequent cDNA expression profiling.

1.2.3.1 Suppression subtractive hybridisation

Suppression subtractive hybridisation (SSH) is one of the most powerful methods for isolating differentially expressed transcripts, which can be used for the generation of subtracted cDNA or genomic DNA libraries. This technique can be used to compare mRNA or genomic DNA populations and to obtain cDNAs of genes exclusively expressed or overexpressed in one population. SSH is based on hybridisation and suppression PCR and combines normalisation and subtraction in a single procedure. The normalisation step equalises the abundance of DNA fragments within the target population. In the subtraction step sequences that are common to both populations are excluded. During this step the cDNA containing the transcripts of interest and the reference cDNA are hybridised and the hybrid sequences are removed. Thus the probability of obtaining low-abundance differentially expressed cDNA or genomic DNA fragments is clearly increased (Diatchenko et al., 1996; Rebrikov et al., 2004).

1.2.3.2 Multi-species microarray

The development of cost-effective high-throughput sequencing technologies has resulted in the accumulation of valuable genome sequence information from an assortment of model organisms including *Arabidopsis thaliana* and rice (*Oryza sativa*) as well as available DNA arrays for a number of species. As for many species standard microarray platforms are not available, arrays of closely related species may be used. Moore et al. (2005) demonstrated that the tomato cDNA array is a viable tool for gene expression profiling in pepper and eggplant. Xu et al. (2008) investigated the wheat transcriptional profile under drought stress with a whole genome rice gene chip platform, which contains over 60 000 oligos based on the

rice genome sequence. Also the available Arabidopsis array has been used to analyse gene expression in other *Brassicacaea* like oil seed rape (Carlsson et al., 2007; Girke et al., 2000). It is assumed that by using arrays of closely related species the foreign RNA will hybridise sufficiently well to the microarray, although there are sequence mismatches between the target RNA and the probes on the array. In a multi-species array RNA or cDNA from each species is hybridised to probes from the same species as well as to probes from other species.

1.3. Bacterial endophytes

Endophytes are microorganisms that spend part or all of their life cycle residing benignly inside host plant tissues. Endophytic bacteria invade living plants causing unapparent and asymptomatic infections but cause no symptoms of disease (Wilson, 1995). They actively colonise plant intercellular plant tissues and establish long-term associations, actually lifelong natural associations (Bacon and Hinton, 2006). These bacteria are not in generally organ specific and have been isolated from roots, stems, leaves, seeds and fruits of many different plants. For example diverse bacteria were isolated from rice, wheat, corn, soybean, sorghum, cotton, diverse grasses as well as from coniferous and deciduous trees (McInroy and Kloepper, 1995; Zinniel et al., 2002; Moore et al. 2006; Izumi et al, 2008; Mano and Morisaki, 2008). Identification of these bacteria revealed a high diversity with a high number of unculturable species.

The endophytes profit from their protected niche with relative little competition from other microorganisms. However, it has been shown that many plants also benefit from endophytic bacteria resulting in plant growth promotion or pathogen defence by antibiosis (Keel et al., 1996; Sharifi-Tehrani et al., 1998; Raaijmakers et al., 1997), competition (Dekkers et al., 2000; Chin-A-Woeng et al., 2000) or induction of systemic resistance (Kloepper and Ryu, 2006). Plant growth promotion due to bacterial endophytes may be achieved by improved nutrient acquisition such as nitrogen fixation (Elbeltagy et al., 2001), phosphate solubilisation (Wakelin et al., 2004) or iron chelation (Costa et al., 1994). Furthermore endophytic bacteria may produce the phytohormones auxin or cytokinine (Timmusk et al., 1999; Mendes et al., 2007). This plant growth promotion is often greater when it is induced by endophytes than by bacteria restricted to the root surface or rhizosphere (Chanway et al., 2000; Conn et al., 1997).

Plant-associated bacteria play a key role in adaptation of the host plant to a changing

environment (Hallmann et al., 1997). For example the plant-growth-promoting rhizobacterium *Burkholderia phytofirmans* Strain PsJN significantly improved cold tolerance of grapevine plantlets (Barka et al., 2006). Also the plant growth-promoting bacterium *Achromobacter piechaudii* strain ARV8 enhanced resistance to water and salt stress in tomato and pepper plants (Mayak et al., 2004 a and 2004b).

Almost all biotic and abiotic stress conditions elicit ethylene synthesis in plants (Bleecker and Kende, 2000). High levels of ethylene usually result in reduced plant growth and is deleterious to plant health except for fruit ripening and the initiation of lateral root growth (Czarny et al. 2006). Plant growth-promoting bacteria may have the ability to synthesise the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which can cleave the ethylene precursor ACC and thereby lowers the level of ethylene in developing or stressed plants (Hontzeas et al., 2006). Thus bacterial endophytes exhibiting ACC deaminase activity can reduce plant stress (Mayak et al. 2004a and 2004b, Grichko and Glick 2001, Burd et al. 1998).

1.4. Aims of the thesis

Pepper is known to be very sensitive to soil water deficit at all stages of its development (González-Dugo et al. 2007). Although water management is extremely important for pepper production only limited data on drought defence mechanisms of pepper plants are available. To develop crop plants with enhanced tolerance to drought stress, a fundamental understanding of physiological and molecular biology networks is essential. It is known that plants respond differently to rapidly applied severe stress and slowly induced water deficit, where plants have the possibility to adapt to the changed environment (McDonald and Davies 1996). Furthermore it has been shown that plants can benefit from endophytic bacteria with respect to growth but also concerning water and osmotic stress resistance (Mayak et al., 2004 a and 2004b).

The aim of the thesis was to study the reactions of pepper plants on slowly increasing water deficit as well as to investigate the growth promoting and stress relief effect of five bacterial endophytes. For this reason bacteria isolated from pepper plants were selected from the strain collection available at the Department of Bioresources at the Austrian Institute of Technology Seibersdorf. Criterion for selection were ACC deaminase activity and/or IAA production. As different organs of a plant have different functions they react differently on

drought and osmotic stress. Therefore all metabolic measurements and gene expression analyses were done in leaves and roots separately. The complexity of the physiological response was taken into account by a wide range of biochemical analysis.

With regard to gene expression the suppression subtractive hybridisation technique was combined with transcription profiling using a microarray. As for many other plants there exists no microarray platform for pepper. Cross-species arrays may be a possibility to overcome this problem. At the Austrian Institute of Technology a large plant EST resource centre and repository is available. Thus it was intended to assemble a drought stress-specific multi-species microarray, which could be tested in parallel.

2. RESULTS AND DISCUSSION

2.1. Organ-specific defence strategies of pepper (*Capsicum annuum* L.) during early phase of water deficit

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ORIGINAL PAPER

Organ-specific defence strategies of pepper (*Capsicum annuum* L.) during early phase of water deficit

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Abstract Drought is one of the major factors that limits crop production and reduces yield. To understand the early response of plants under nearly natural conditions, pepper plants (*Capsicum annuum* L.) were grown in a greenhouse and stressed by withholding water for 1 week. Plants adapted to the decreasing water content of the soil by adjustment of their osmotic potential in root tissue. As a consequence of drought, strong accumulation of raffinose, glucose, galactinol and proline was detected in the roots. In contrast, in leaves the levels of fructose, sucrose and also galactinol increased. Due to the water deficit cadaverine, putrescine, spermidine and spermine accumulated in leaves, whereas the concentration of polyamines was reduced in roots. To study the molecular basis of these responses, a combined approach of suppression subtractive hybridisation and microarray technique was performed on the same material. A total of 109 unique ESTs were detected as responsive to drought, while additional 286 ESTs were selected from the bulk of rare transcripts on the array. The metabolic profiles of stressed pepper plants are discussed with respect to the transcriptomic changes detected, while attention is given to the differences between defence strategies of roots and leaves.

Keywords Drought · Gene expression · Metabolic · Osmotic potential · Organ-specific response

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Introduction

Drought is perhaps the most common abiotic stress limiting crop productivity world-wide. For a better understanding of stress tolerance the elucidation of the plant's responses to various stages and levels of severity of drought is important. One prominent response to drought stress is the accumulation of the manifold substances that modulate the osmotic level of the plant cell's cytosol and the vacuoles, with the aim to counteract the loss of turgor (Cushman 2001). These osmolytes include amino acids, such as proline and quaternary ammonium compounds, glycine betaine, hydrophilic proteins (e.g., late embryogenesis abundant proteins), carbohydrates (e.g., fructan, sucrose), and polyols (e.g., pinitol, mannitol) (Chaves et al. 2003). Environmental stresses have also been demonstrated to increase the level of polyamines (Liu et al. 2004). Besides their function as osmolytes these organic compounds may also be accumulated to maintain the structure and activity of enzymes and other proteins and to protect membranes from damage by reactive oxygen species (ROS) (Chaves et al. 2003). In addition, it is known that sucrose and other sugars regulate the expression of many genes involved in photosynthesis, respiration, nitrogen and secondary metabolism as well as in pathogen defence (Pinheiro et al. 2001).

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The experiments of Seki et al. (2001, 2002) demonstrated the validity of gene microarray technology for the monitoring of simultaneous changes in the expression level of a large number of genes that together comprise the complex drought tolerance trait. Early-response genes are induced very quickly, within minutes, and often transiently, whereas delayed-response genes, which constitute the vast majority of the stress-responsive genes, are activated by stress more slowly, within hours, and their expression is often sustained (Zhu 2002). In addition, the degree of changes in gene expression may vary depending on stress intensity, as experiments on loblolly pine by Watkinson et al. (2003) have suggested. Kacperska (2004) distinguished moderate and severe stress and the corresponding overall plant responses. A primary response to moderate stress would be the disturbance of water balance affecting the interaction between cell wall and plasma membrane that results in the activation of receptor-like kinases, including wall-associated kinases, cytoskeleton-related mechanosensors, stretch-dependent ion channels and redox-mediated systems. In contrast, severe or suddenly acting stressors are sensed by membrane destabilisation, which leads in turn to triggering of phospholipid signalling.

We have been interested in the response of pepper (*Capsicum annuum* L.) to early moderate drought stress, since pepper is considered as one of the most sensitive crops to soil water deficit (González-Dugo et al. 2007). Especially drought stress during early growth stages might result in reduced plant size and number of blossoms and fruits (Rylski and Spigelman 1982). However, despite that water management in pepper is extremely important at all stages of plant development due to its influence on stand establishment, fungal problems and fruit set and quality, there are very limited data on mechanisms of drought avoidance or defence in pepper. Therefore, we studied the responses of greenhouse-grown, potted pepper plants to increasing moderate drought stress. In addition to biochemical measurements, we looked at the molecular biology background of pepper responses. The approach we used combines the suppression subtractive hybridization technique (SSH), which has been proved to be a powerful tool for enrichment of induced genes (Way et al. 2005, Ouyang et al. 2007), with transcription profiling. Furthermore, the responses of leaves and roots were compared to clarify pepper plant's defence strategy against drought.

Materials and methods

Plant materials, growth conditions and stress treatment

Seeds of *Capsicum annuum* L. cv. Ziegenhorn Bello were germinated on water soaked filter paper and afterwards placed into 0.5-l container with commercial soil and perlite (1:1 v/v). Four plants per pot were kept in a growth chamber set to a 12-h photoperiod at a constant temperature of 24°C and 80% relative humidity. Upon full expansion of their fourth leaf plants were transferred into a greenhouse with natural light conditions and 10 h dark period. Temperature was kept at 18–22°C and relative air humidity varied between 60 and 80% during day and night, respectively. Upon full development of 6–7 leaves of the main shoot watering of the pots was suspended for 1 week, whereas control plants were further watered to maintain the water content of the substrate between 40 and 60% (Fig. 1). Water content of the substrate was measured using a Theta Probe ML2 (Delta-T Device LTD.) connected to an Infield 7 Data Logger (UMS GmbH Munich).

The youngest fully expanded leaves were harvested at 11 am at the end of the drought stress period (day 7) and immediately frozen in liquid nitrogen. Roots were washed with tap water and also frozen immediately. Leaves and roots of seven pots were pooled and ground to fine powder in a Retsch MM 200 automatic grinder. Thus, one sample (replicate) consisted of 28 plants (1 leaf/all roots per plant, 4 plants per pot, 7 pots per replicate). Six replicate samples each of leaves and roots of stressed and control plants were stored at −80°C until analysis.

Water content of leaves

The second youngest fully expanded leaves of all four plants of each pot were harvested together and weighted. After drying at 60°C for 24 h their weight was determined again and the relative water content calculated.

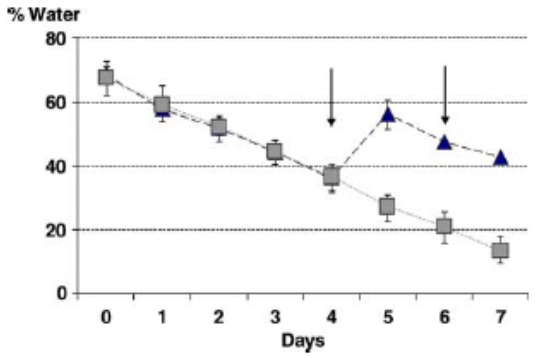

Fig. 1 Water content of the substrate in that pepper plants were grown in greenhouse conditions. *Arrows* indicate days of watering of the control plants. *Triangles* control plants, *squares* drought stressed plants

Osmotic potential

For analysis of the osmotic potential, Ψ, approximately 500 mg fine powder of leaves and roots, respectively, were thawed and centrifuged twice for 5 min at maximum speed. The osmotic potential of the supernatant was determined with a digital micro-osmometer (VOGEL, Giessen, Germany) by measuring the freezing point depression, which is directly proportional to the osmolality (solute concentration expressed in mOsmol kg^{-1}). A multiplication factor (−2.4789) was used to convert osmolality (Osm kg^{-1}) into osmotic potential (Ψ, Pa) at 25°C (Prewein et al. 2004). The measurements were done twice with six replicates each.

Metabolite analyses

Contents of soluble carbohydrates, polyols and free polyamines were performed as described previously (Oufir et al. 2008). Proline and related compounds analogues were quantified by Oufir et al. (2009).

Statistical analysis

Data on leaf water content, osmotic potential and levels of all analysed osmolytes were analysed using analysis of variance (ANOVA) and Student's *t* test.

Preparation of subtracted cDNA libraries

Total RNA was extracted via a modified method by Chang et al. (1993) according to Fluch et al. (2008). Poly-A^+ RNA was isolated using a Dynabeads mRNA Purification Kit (Dynal Biotech ASA, Oslo Norway).

Subsequently, four SSH cDNA libraries were constructed using the PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, USA). The forward-subtracted libraries from both leaves and roots used cDNA of stressed plants as tester and cDNA of well-watered control plants as drivers. For the corresponding reverse libraries cDNAs were used reciprocally. The subtracted cDNA fragments were cloned into *E. coli* using the TOPO TA Cloning Kit and chemically competent *E. coli* cells strain TOP 10 (Invitrogen) as described by the manufacturer. For each of the four libraries 960 clones were picked.

Amplification of cDNA inserts

Circular DNA of the clones was amplified using the TempliPhi DNA Sequencing Template Amplification Kit (Amersham Biosciences) and stored at −20°C until use. These amplified products were used as template for further PCR amplification using M13 primers (M13 forward: 5′-GTAAAACGACGGCCAG-3′, M13 reverse: 5′-CAGGAAACAGCTATGAC-3′). The insert size of 3,231 clones (controlled by standard agarose gel electrophoresis) was 200–800 bp. Clones containing no or more than one insert were removed from further investigation. All PCR-products were spotted onto microarray slides.

Additional ESTs and genes

As positive controls, fragments of two putative proline synthesis-related genes were isolated via PCR amplification from pepper cDNA and included in the microarray. These genes show high similarities to genes of tomato and potato encoding Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) and Δ^1-pyrroline-5-carboxylate reductase (*P5CR*). The primers designed using Primer 3 software (http://frodo.wi.mit.edu) were: P5CR-forward, 5′-GAAAGCATAGCTAGAGGTGTGG-3′; P5CR-reverse, 5′-CAGCTTCTCATCAGCTTTCCA-3′; P5CS-forward, 5′-ATGCTCGAGAGATGGCAGTT-3′ and P5CS-reverse, 5′-CCTCCTTCCCTCCTTTCAAC-3′.

As negative controls, cDNA of human genes were included in multiple copies in an ordered distribution across the microarray.

Preparation of microarray slides

From the cDNA clones and additional fragments included on the microarray, 20 µl PCR product was lyophilised and re-suspended in 10 µl spotting buffer (3× SSC and 1.5 M betaine). PCR products were spotted onto glass slides in duplicates as described by Fluch et al. (2008).

Hybridisation with not subtracted cDNA

To analyse gene expression in leaves and roots four slides each were hybridised with labelled cDNA of stressed and control plants of four different biological replicates. Half of the slides were used as dye swap with reciprocal labelled cDNAs.

RNA of leaf and root tissue was isolated as described above. Labelled cDNA was prepared using the template-switch-PCR method according to Petalidis et al. (2003) with 500 ng total RNA. Double-stranded cDNA was purified using a QIAquick purification Kit (Quiagen). Labelling with Cy3- and Cy5-dyes (GE Life Science) was performed using BioPrime Array CGH Genomic Labeling System (Invitrogen). The mixture of Cy3- and Cy5-labelled cDNA was denatured for 3 min at 95°C and mixed with 20 µl of 4× hybridisation mix (GE Life Science) and 40 µl formamide. The slides were hybridised at 42°C overnight and then washed in 0.1% SDS in 1× SSC at 50°C for 15 min followed by 0.1% SDS in 0.1× SSC for 10 min at

room temperature. After two washes with 0.2× SSC for 5 min at room temperature slides were dried with pressure air.

Hybridisation with forward and reverse subtracted libraries

Two slides each were hybridised with subtracted cDNA fragments prepared from leaf or root tissue including a dye swap with reciprocally labelled cDNAs. Adaptors were removed from subtracted cDNAs by digestion with *Rsa*I (BioLabs Inc.). Following separation on 1% agarose gel the PCR fragments were excised, purified (QIAquick Gel Extraction Kit, Quiagen) and labelled using the BioPrime Array CGH Genomic Labeling System (Invitrogen).

Microarray data analysis

The slides were scanned with an LS Reloaded scanner (Tecan) and the resulting images were processed with GenPixPro 6.0 software (Axon Instruments Inc.). A spatial and intensity-dependent (LOWESS) normalisation method was employed to normalise the ratio values of the raw data, which were afterwards statistically analysed using LIMMA software package in R (Smyth 2004) from the Bioconductor project (http://www.bioconductor.org). The cutoff value was set to $p < 0.01$. The complete expression data set is available under platform ID number GPL9451, series accession number GSE18627 in GEO at NCBI.

EST sequence analysis

All cDNAs identified as differentially expressed were sequenced on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) after purification and labelling with "Big Dye" terminator cycle sequencing kit (PE Applied Biosystems). The sequences were trimmed from vector and adaptor regions and annotated by performing sequence similarity searches against NCBI nr database using BLASTX program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) with a cutoff value of ≤1e-15. Functional classification of these genes was based on MIPS functional catalogue (http://mips.gsf.de/projects/funcat).

Real-time RT-PCR

SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, USA) was used to transcribe 2.5 µg total plant RNA into cDNA. A pepper translation initiation factor eIF-3b was taken as inner standard for quantification of the transcript abundance. All primers were designed using the Primer3 program (http://frodo.wi.mit.edu) and are listed in Online Resource S1.

Real-time PCR was carried out in a BioRad iCycler using SYBR Green qPCR Supermix-UDG (Invitrogen, Carlsbad, USA). PCR was performed starting with 2 min at 50°C 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 last cycle to exclude any influence of primer–dimer pairs. Cycle numbers at which the fluorescence passed the cycle threshold (Ct) were further analysed using the ΔΔCt-method and REST© (Relative Expression Software Tool) (Pfaffl et al. 2002).

For the amplification of rare mRNA species, 1 µg of total RNA was reverse transcribed using the iScript Select cDNA Synthesis Kit (BioRad) with an oligo dT primer and enhancer according to the manufacturer's protocol. PCR-products from templates of leaves of unstressed and stressed plants were compared by standard agarose gel electrophoresis.

Results

Plant physiology

Water was withheld from pepper plants for 1 week to simulate gradual development of drought stress. During this time soil water content decreased to nearly 10%, whereas soil water content of the control plants was kept between 40 and 50% by watering (Fig. 1). At the end of this period the stressed plants showed first leaf wilting symptoms but no change in leaf water content could be determined. The water deficit resulted in a decrease of osmotic potential Ψ in roots from -0.44 ± 0.012 to -0.53 ± 0.019 MPa. Similarly, in leaves a significant though less pronounced decline could be measured ranging from -0.73 ± 0.008 to -0.76 ± 0.007 MPa.

Biochemical analyses

The levels of arabinose, fructose, galactose, glucose, raffinose, sucrose, trehalose and xylose varied to different extents depending on organ and treatment considered (Fig. 2). In stressed plants contents of fructose and sucrose increased significantly in leaves, whereas high amounts of glucose and raffinose accumulated in roots. A decrease was determined for arabinose in roots and trehalose in leaves. In none of the tissues significant changes were observed for the concentrations of galactose and xylose under drought.

Among four polyols evaluated only the level of galactinol increased in leaves and roots due to stress treatment (Fig. 3). The amounts of inositol, pinitol and mannitol were not significantly altered.

Water deficiency also significantly affected the contents of proline and its analogues hydroxyproline and

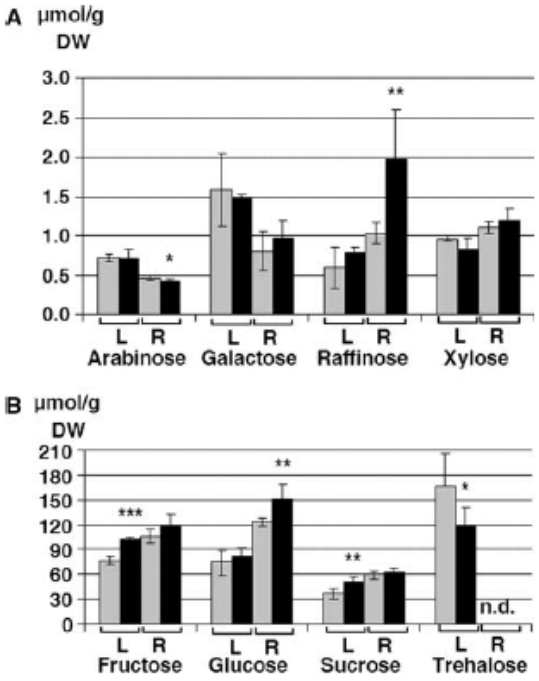

Fig. 2 Carbohydrate contents in leaves (*L*) and roots (*R*) of control (*gray-coloured boxes*) and water-deficient (*black*) plants ($n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *n.d.* not detectable. *Bars* indicate SD)

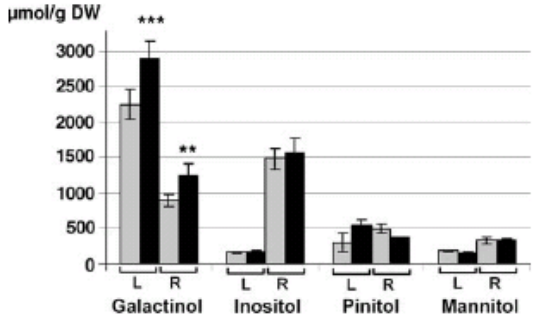

Fig. 3 Polyol contents in leaves (*L*) and roots (*R*) of control (*gray-coloured boxes*) and water-deficient (*black*) plants ($n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *Bars* indicate SD)

methylproline (Fig. 4). The proline level increased nearly four times in root tissue, whereas it was reduced in leaves. Hydroxyproline accumulated in leaves and also in roots but these changes were less pronounced as it was in case of proline. Although concentrations of methylproline were generally low, a significant increase was measured in roots of stressed plants.

Regarding polyamines, drought stress resulted in elevated concentrations of all analysed polyamines in leaves.

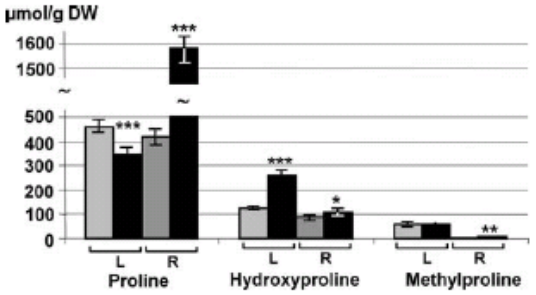

Fig. 4 Proline and its analogue contents in leaves (*L*) and roots (*R*) of control (*gray-coloured boxes*) and water-deficient (*black*) plants ($n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *Bars* indicate SD)

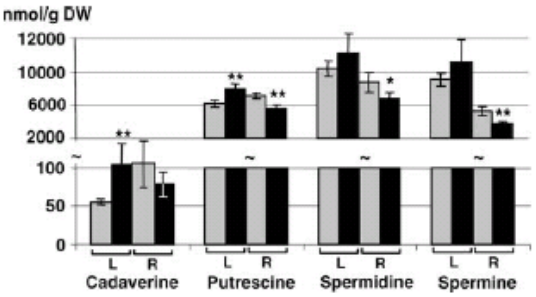

Fig. 5 Polyamine contents in leaves (*L*) and roots (*R*) of control (*gray-coloured boxes*) and water-deficient (*black*) plants ($n = 4$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *Bars* indicate SD)

However, significant change was only observed for cadaverine and putrescine (Fig. 5). In contrast, levels of putrescine, spermidine and spermine decreased significantly in root tissue.

SSH–microarray hybridisation

Four subtracted cDNA libraries from leaves and roots exposed to water deficit and control pepper plants were created. These resulted in 3,231 clones of 200–800 bp, which were used for preparation of microarrays as described. After co-hybridisation of the slides with cDNA from control and drought-exposed pepper plants, background was subtracted. However, ~60% of all probes from leaves and ~72% from roots were flagged as non-detectable due to their too low signal strength. Similar result was obtained, when a different labelling technique (SuperScript Direct cDNA Labelling, Invitrogen) was used (data not shown). Nevertheless, in total 109 unique differentially expressed cDNA clones ($p \leq 0.01$, fold change ≥ 2) from leaves and roots were identified (Online Resource S2). More than 80 and 90% of the clones up-regulated in both leaves and roots, (respectively) were isolated by forward subtraction,

and the proportion of down-regulated genes in the reverse libraries was similar.

One of the largest expression changes in this experiment was measured for clone FG981192, which encodes for a hypothetical protein and shows similarities to the desiccation responsive protein RD29B from *Arabidopsis*. This clone was >40-fold up-regulated in leaves and >30-fold up-regulated in roots. Among other ESTs highly up-regulated in roots and leaves were FG981257 and FG981267, which both showed no similarities to any other database record.

The identified drought responsive genes belong to representatives of many different processes within the global metabolism of the plant cell (Fig. 6). A large fraction (12.8%) of the identified ESTs is probably involved in energy metabolism. This category included 12 different chlorophyll a/b-binding and light harvesting proteins, which all were down-regulated in leaves. Further, 13.8% of the ESTs are possibly involved in metabolic pathways like carbohydrate, proline, ethylene or amino acid metabolism. The third largest group of the differently expressed ESTs might be related to cell rescue (Table 1) and included an up-regulated dehydrin (FG981234), peroxidases (FG981200, FG981245) and down-regulated heat-shock proteins (FG981253, FG981279) as well as a gene encoding for an osmotin-like protein (FG981288). Several genes could be assigned to the category cellular communication and signal transduction mechanism. Among these was a highly induced Ca-binding protein FG981179 as well as several differentially expressed protein kinases (FG981177, FG981233, FG981244, FG981254, FG981210). In addition divers putative transcription factors were identified, most of them belonging to the zinc finger family. Among proteins with a binding function the two putative RNA-binding genes FG981176 and FG981178 were found to be strongly induced in leaves as well as in roots. Only 18 ESTs were significantly up-regulated and nine down-regulated in both leaves and roots (Fig. 7, Online Resource S3).

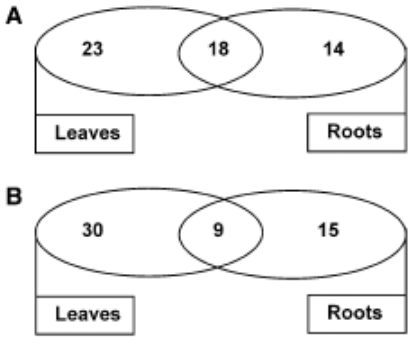

Fig. 6 Number of genes significantly ($p \leq 0.01$, ≥twofold) up-regulated (**a**) or down-regulated (**b**) in leaves and roots

Not a single EST clone was regulated into opposite direction within the two organs.

Changes in mRNA abundance detected by microarray analysis were validated by quantitative real-time RT-PCR. Expression pattern of four selected genes, in both roots and leaves, did coincide using the two techniques (Fig. 8).

Gene expression: hybridisation with subtracted cDNA

The clones corresponding to spots with very weak or no hybridization signal in microarray analysis were analysed further. Since a technical error was excluded (all slides showed similar patterns even when different labelling techniques were used, data not shown), microarray hybridizations were repeated but using the subtracted amplicons as targets. As a result, additional 286 ESTs from leaves and roots, previously yielding no signal, could be clearly assigned to a specific library ($p < 0.01$, log2 ratio > |1.0|). A subset of 25 EST clones were sequenced, 23 of them annotated and assigned to functional classes (Online Resource S3). Five ESTs were classified to metabolism category, four ESTs related to transcription and three encoded for proteins with a binding function. Seven ESTs were assigned to other functional classes with one or two in each. The rest of the ESTs belonged to the group of unknown function and provided no further information.

To reveal the behaviour of low-signal yielding rare transcripts identified by the SSH-microarray combined approach, qRT-PCR was performed. However, reverse transcription and amplification failed; therefore, a different transcriptase with an enhancer (iScript Select cDNA Synthesis Kit, BioRad) was used. Expression of five ESTs isolated by forward subtraction from leaves was shown to be clearly up-regulated upon water deficit (Fig. 9), thus confirming the result of the SSH-microarray hybridisation.

Discussion

In this study pepper plants growing in a greenhouse were stressed by withholding water for 1 week, and subsequently accumulation of compatible solutes was analysed. In general, various osmolytes accumulated in affected plants, which were different in roots and leaves and amplitudes of changes were more pronounced in roots. Modifications of carbohydrate metabolism in stressed leaves were manifested by significant accumulation of sucrose and fructose. The increase of fructose, sucrose and other soluble carbohydrates is a well-known effect in drought stressed plants (Kerepesi and Galiba 2000; Pinheiro et al. 2001; Taji et al. 2002; Trouverie et al. 2003), although the reduction of leaf sucrose due to drought is

Table 1 Gene expression of selected ESTs

Accession No.	Annotation	e value	Leaves (*x*-fold change)	Roots (*x*-fold change)
ESTs possibly involved in cell rescue, defence and virulence				
FG981200	Cationic peroxidase	2e-31	**2.19**	1.69
FG981272	Chitinase class II	2e-56	**4.16**	**21.78**
FG981234	Dehydrin	4e-06	**10.55**	**33.40**
FG981300	Disease resistance-responsive family protein (dirigent-like protein)	2e-06	ns	**0.44**
FG981253	Heat shock protein 70	6e-74	**0.40**	0.57
FG981279	Heat shock protein 70-3	3e-59	**0.33**	**0.28**
FG981245	Ascorbate peroxidase	1e-43	ns	**2.48**
FG981288	Osmotin-like protein	2e-44	ns	**0.39**
FG981273	Pathogenesis-related protein 10	1e-30	ns	**0.50**
FK265367	Pathogenesis-related protein 8	4e-48	**5.07**	ns
FG981228	Pathogenesis-related protein 8	1e-94	ns	**6.16**
FG981251	Heat shock protein	6e-77	ns	For
FG981280	Unnamed protein product (Harpin-induced protein 1 (Hin1))	1e-38	ns	Rev
ESTs possibly involved in cellular communication/signal transduction mechanism				
FG981179	Ca-binding protein	6e-49	**2.59**	**9.56**
FG981229	Hypothetical protein (EF-hand, calcium binding motif)	3e-16	**2.65**	ns
FG981261	Hypothetical protein with Ca-binding motif	3e-16	**2.65**	ns
FG981177	Leucine-rich repeat protein kinase, putative	7e-10	**3.32**	ns
FG981233	PERK1-like protein kinase	2e-13	**4.13**	ns
FG981244	Putative protein kinase	2e-40	ns	**2.01**
FG981254	Putative receptor protein kinase PERK1	2e-66	ns	**7.25**
FG981210	Receptor protein kinase	1e-66	**0.22**	ns
FG981202	ADK/ATPADK1 (adenosine kinase); nucleotide kinase	6e-81	For	ns
ESTs encoding for transcription factors or possibly involved in transcription				
FG981274	BTB/POZ; MATH	2e-27	**2.53**	ns
FG981252	Unnamed protein product, DHHC zinc finger domain	2e-12	**0.41**	ns
FG981299	RWP-RK domain-containing protein	2e-13	ns	**0.50**
FG981285	Transcription factor WRKY14	5e-25	ns	**0.30**
FG981258	Hypothetical protein (Putative zinc fingers with GTPase activating proteins (GAPs) towards the small GTPase, Arf.)	1e-67	**4.95**	**28.17**
FG981203	Unnamed protein product; Zinc finger domain in Ran-binding proteins (RanBPs)	3e-59	For	ns
FG981249	Unnamed protein product Uncharacterized conserved protein, contains RING Zn-finger	5e-22	ns	For
FG981255	Unnamed protein product, SWIB/MDM2 domain	3e-39	ns	For
ESTs involved in proline metabolism				
CO907770	Putative P5CS	0	1.4	1.6
TC4099	Putative P5CR	0	1.5	1.5
FG981287	Proline oxidase/dehydrogenase 2	6e-83	0.60	**0.28**

Reliability of the BLAST search is given for each clone by the e value. Bold values correspond to $p \leq 0.01$ and fold change ≥ 2

ns not significant change, *For* clones with expression below the detection limit are assigned to a specific library as forward subtracted, most probably up-regulated genes, *Rev* reverse subtracted, most probably down-regulated gene

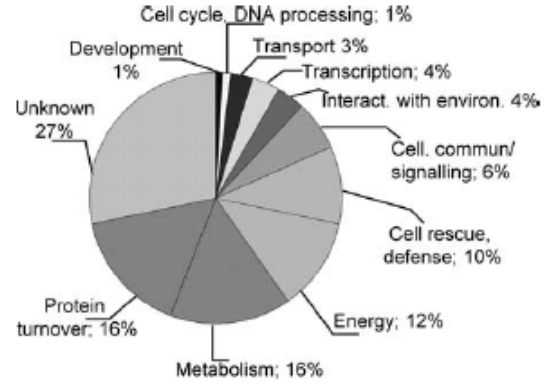

Fig. 7 Distribution of 109 unique drought-responsive ESTs based on MIPS functional categories. The percentage of gene transcripts in each group is stated

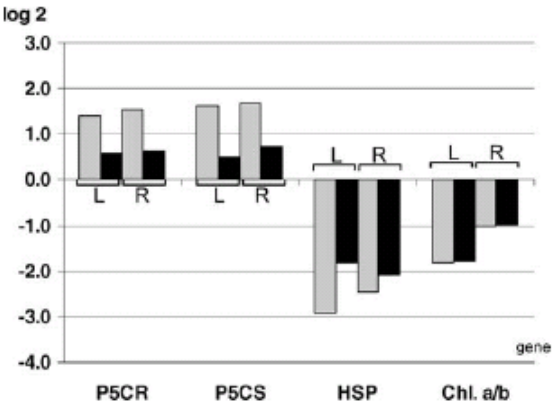

Fig. 8 Verification of microarray results (*black-coloured boxes*) by real-time RT-PCR (*gray*) ($n = 4$, ns = not significant). The genes analyses were done on leaves (*L*) and roots (*R*). *P5CS* (*TC4099*) putative Δ^1-pyrroline-5-carboxylate synthetase, *P5CR* (*CO907770*) putative Δ^1-pyrroline-5-carboxylate reductase, *HSP* (*FG981253*) gene homologue to heat shock protein, *Chl. a/b* (*FG981211*) gene homologue to chlorophyll a/b-binding protein

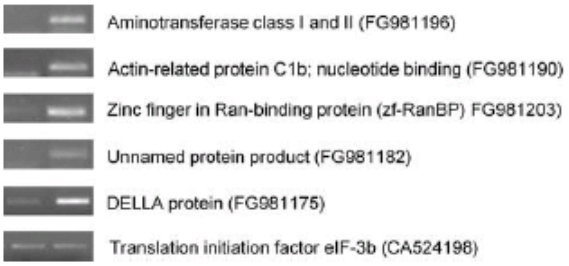

Fig. 9 RT-PCR of selected genes significantly more abundant in the leaf forward library. *Left* unstressed plants, *right* stressed plants, amplification products loaded on electrophoresis gel were 10 µl for the differentially expressed genes and 3 µl for the relatively highly expressed translation initiation factor eIF-3b

reported as well (Liu et al. 2003). Trehalose levels decreased significantly in leaves and could not be detected in roots at all. In contrast to results with drought stressed wheat cultivars (El-Bashiti et al. 2005), this disaccharide obviously is not accumulated as an osmoprotectant in pepper in neither of the analysed organs.

Significant and pronounced accumulation of both raffinose and glucose was observed in stressed roots (in contrast to leaves). This confirms that raffinose may be involved in abiotic stress tolerance in plants and may function as osmoprotectant under drought stress (Taji et al. 2002). The trisaccharide raffinose may be the more effective in membrane stabilization than either disaccharide sucrose or monosaccharide glucose (Taji et al. 2002) and appears to contribute to the defence strategy of pepper roots during early drought. Similar protective effect is suggested for galactinol (Taji et al. 2002), which was the only analysed polyol that accumulated significantly in both organ types. Beside their role in energy metabolism and osmotic adjustment, both galactinol and raffinose are suggested to act as scavengers of reactive oxygen molecules to protect plant cells from oxidative damage (Nishizawa et al. 2008).

Proline accumulation is a very common response of plants to drought conditions. In stressed pepper proline levels increased almost four times in roots; however, decreased in leaves. At the same time, elevated contents of hydroxyproline, a derivative of proline through hydroxylation found mostly in hydroxyproline-rich proteins in plants, was observed in both leaves and roots. Since proline hydroxylation follows proline incorporation into synthesis of cell wall proteins (Golan-Goldhirsh et al. 1990; Ueda et al. 2007), incorporation of free proline after hydroxylation to structural proteins of the cell wall might be induced in pepper in response to drought, more in leaves than in roots stress (Fig. 4). Increased synthesis of cell wall components could be assigned to an adaptive response to mechanical and injury stresses caused by the initial drought stress (Ueda et al. 2007). In addition, levels of another proline-derived compound, methylproline, was elevated in pepper roots upon drought (Fig. 4.) and might serve as even more potent osmoprotectant than proline (Hanson et al. 1994).

In leaves, elevated levels of cadaverine and putrescine were detected (in contrast to roots) that has been shown to induce closure of stomata and strongly inhibit their opening (Liu et al. 2000a). Moreover, cadaverine was demonstrated to protect DNA from oxidative degradation in vitro, and with spermine it was suggested also to act as a free radical scavenger (Ha et al. 1998; Kuznetsov et al. 2007). Involvement of polyamines in response to environmental stresses is also confirmed by the enhanced drought tolerance of plants over-expressing genes involved in the synthesis of putrescine, spermidine and spermine (Capell et al.

2004). Since the levels of polyamines were rather low, they might be involved in stress protection of pepper leaves rather than (osmotic) adaptation to drought.

Gene expression changes with respect to drought

The combination of SSH and microarray technique resulted in 109 unique ESTs, the altered expression of which can be ascribed to the accommodation process at very early stage of drought stress. The complexity of the physiological response of the pepper plants to given condition is reflected in the broad spectrum of functional classes of identified gene representatives. Many of these have previously been described in studies with severe water or salt stress in different plant species (Seki et al. 2002; Way et al. 2005; Ouyang et al. 2007).

There were several cell rescue- and defence-related genes induced by drought in pepper, including a gene representative for dehydrin, a class II chitinase as well as lysozyme-like class III chitinase (pathogenesis related protein PR-8). Genes encoding for peroxidases were significantly up-regulated in roots (ascorbate peroxidase) and in both roots and leaves (cationic peroxidase). The corresponding enzymes catalyze the oxidation of a wide variety of substrates in the presence of H_2O_2 as an oxidizing agent. Formation of reactive oxygen molecules during drought condition is the consequence of the reduced activity of photosystem II (Reddy et al. 2004) and could be related to the observed down-regulation of several pepper genes encoding chlorophyll a/b binding proteins as well as a ribulose 1,5 bisphosphate carboxylase/oxygenase (RuBisCo), small subunit protein in pepper leaves. In addition, two ESTs encoding for HSP70 and HSP70-3 (Table 1), possibly assisting by integration of the mature proteins of the light harvesting complex of photosystem II into thylakoid membranes (Yalovsky et al. 1992), were repressed in pepper during drought. Such proteins have previously been described as down-regulated under early salt stress in tomato roots (Ouyang et al. 2007).

Drought resulted in elevated transcript levels of EST encoding for 1-aminocyclopropane-1-carboxylate oxidase in pepper leaves, which catalyses the final step of ethylene biosynthesis (Bleecker and Kende 2000). Ethylene is elicited and acts as signal mediator under almost all biotic and abiotic stress conditions including drought (Bleecker and Kende 2000; Liu et al. 2000b). Together with many other molecules (including, e.g., reactive oxygen molecules) ethylene can initiate phosphoprotein cascades, which afterwards may activate transcription factors regulating stress responsive genes (Hu et al. 2006; Lu et al. 2007). Indeed, several pepper protein kinases and receptor protein kinases (Table 1; Online Resource S2) as well as different transcription factors (Table 1; Online Resource S3) were differentially expressed (mostly induced) under drought, especially in leaves. Interestingly, mainly transcription factors containing a zinc-finger domain were identified. According to Kacperska (2004) the expression of protein kinases reflects to moderate stress of pepper plants, whereas ESTs involved in phospholipid signalling (there were none detected in this experiment in neither leaves nor roots) are usually a sign of severe and sudden stress.

The simultaneous analysis of transcriptional and metabolic profiles in this study provides an opportunity to search for matches between metabolic genes and their metabolites in stressed pepper plants. This applies for the accumulation of proline (Fig. 4) and the expression of the proline metabolism related genes encoding for pyrroline 5-carboxylate synthetase (P5CS), pyrroline 5-carboxylate reductase (P5CR) and the proline dehydrogenase (PDH) (Hare et al. 1999; Kavi Kishor et al. 2005) (Table 1). Despite contrasting proline accumulation pattern in drought-affected leaves and roots, the corresponding transcript levels for P5CS, P5CR and PDH were very similar in the two organ types. The data obtained in roots, suggesting elevated proline accumulation at both transcript and protein levels as well as repressed catabolism at transcript level, are in agreement with literary reports on proline metabolism under drought stress in different plant species (Su and Wu 2004; Molinari et al. 2004; De Ronde et al. 2004; Kavi Kishor et al. 2005). However, the transcript profile detected does not correspond to decreased metabolite concentration observed in leaves. This observation might be explained by transfer of proline into roots (Girousse et al. 1996). Laher et al. (2003) suggested that osmotically induced proline accumulation is a symptomatic disorder induced by dehydration rather than a prerequisite for immediate water deficit tolerance.

In plants, proline can also be synthesised by an alternative path from ornithine via ornithine aminotransferase (Delauney and Verma 1993), while this pathway competes for the substrate (ornithine) with the pathway for synthesis of polyamines (Theiss et al. 2002). Hence, decreased levels of putrescine, spermidine and spermine (Fig. 5) might reflect the preferential proline synthesis in roots (through both known pathways). Elevated proline synthesis in roots in combination with transport of proline from leaves to roots might function as a strategy of plant adaptation in the pepper tissue primarily exposed to drought. However, since no specific ESTs regarding metabolism of carbohydrates, polyols or polyamines were isolated in SSH-microarray assay, this proposal needs to be further analysed and proven.

Apparently, the difference of the transcriptomes of the control and water deficient plants was small, thus resulting in many not- or only slightly differentially expressed genes. In addition SSH is a technique especially designed for the

detection of rare transcripts and low-expression mRNAs (Diatchenko et al. 1996). These low abundance mRNAs are more difficult to examine using microarrays than high abundance mRNAs due to the effect of concentration on hybridisation kinetics and signal-to-noise ratios (Rondeau et al. 2005). It was assumed that the large number of probes showing no or too weak signals (up to 60 and 72% of spotted clones in leaves and roots, respectively) can be ascribed to the isolation of many rare transcripts, which failed to be detected by microarray analysis. Similar observations have been made by several authors in different systems (Hida et al. 2000; Boeuf et al. 2001; Mercke et al. 2004). After the slides had been hybridised with labelled subtracted cDNA, several ESTs could be assigned to certain libraries, which gives an indication of their different expression. However, hybridisation of the microarray with subtracted cDNA may result in more false positives and results need critical interpretation. Nevertheless, a number of clones were identified by the combined approach of SSH and microarray hybridizations that would have not been detected by the use of these methods separately.

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Supplement 1
Table S1: Primers used in gene expression analyses by real-time RT-PCR and RT-PCR

Code, Gene name, encoded proteins	GenBank accession no. NCBI / TIGR	Primer forward / Primer reverse
Translation initiation factor eIF-3b	CA524198	5′-TGTATTGGCAAAGCAATGGA-3′ 5′-TGTTGTCAAGCTCCAAAACCT-3′
Homologue to chlorophyll a/b-binding protein	FG981211	5′-CCATGTTCTCCATGTTTGGAT-3′ 5′-ACTTTTCCGGGAACGAAGTT-3′
Homologue to heat shock protein	FG981253	5′-CTTAGCCCGGATGACAAGAA-3′ 5′-GGGATTACAGATGCCCTCAA-3′
Putative Δ^1-pyrroline-5-carboxylate reductase (*P5CR*)	CO907770	5′-GTGTCGCAGTTAAGGCCAAT-3′ 5′-ACCAACAGCAGAGGGAGTGT-3′
Putative Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*)	TC4099	5′-GCTGCTCAACAAGCTGGATA-3′ 5′-AGCAAGCTCCGTCCTCTTTA-3′
DELLA protein	FG981175	5′-CGACGGGTGTATGATGTT-3′ 5′- ACTCGCTACTTGACTCGGTGA-3′
unnamed protein product	FG981182	5′-CCACCAATTGGGTAATTGAACT-3′ 5′-TCCCCTATGGCAGAAATTACAT-3′
ARPC1b (actin-related protein C1b); nucleotide binding	FG981190	5′-GCCTCCTATTTCCCACCATAA-3′ 5′-ACTCCGATAAATCTGCCTGGT-3′
unnamed protein product; Zinc finger in Ran-binding protein and others (zf-RanBP)	FG981203	5′-TGGAGACCTATAGCCACCATTT-3′ 5′-AAGTCCTCGAAGGGGTTATTCT-3′
unnamed protein product (Aminotransferase class I and II)	FG981196	5′-GACGTTGTTGTTCACAGCATTT-3′ 5′-GCCTTGGTGTAGATCCATCATT-3′

Supplement 2

Table S2: Genes significantly differentially expressed under mild drought stress (Reliability of the BLAST search is given for each clone by the e value. Bold values correspond to p ≤0.01 and fold change ≥2, not significant change is indicated as n.s.)

Accession No.	ANNOTATION	e-value	Leaves x-fold change	Roots x-fold change
Metabolism				
FG981292	1,3-beta-glucan glucanohydrolase	5e-49	n.s.	**0.34**
FG981195	1-aminocyclopropane-1-carboxylate oxidase	4e-16	**4.05**	n.s.
FG981275	mitochondrial glycine decarboxylase complex T-protein	2e-21	**0.37**	n.s.
FG981180	ATEP3 (Arabidopsis thaliana chitinase class IV); chitinase	6e-41	n.s.	**0.44**
FG981199	ATMGL; catalytic/ methionine gamma-lyase	4e-68	**5.38**	n.s.
FG981198	ATMGL; catalytic/ methionine gamma-lyase	1e-29	**2.16**	n.s.
FG981277	putative phosphatidylglycerolphosphate synthase	2e-26	n.s.	**2.11**
FG981282	formate dehydrogenase	1e-65	**2.21**	n.s.
FG981187	formate dehydrogenase, mitochondrial precursor	4e-66	**2.00**	n.s.
FG981213	glycolytic glyceraldehyde 3-phosphate dehydrogenase	2e-32	**0.50**	n.s.
FG981204	nicotianamine synthase	1e-23	n.s.	**0.40**
FG981287	proline oxidase/dehydrogenase 2	6e-83	0.60	**0.28**
CO907770	putative P5CS	0	1.40	1.60
TC4099	putative P5CR	0	1.50	1.50
FG981191	ribulose 1,5 bisphosphate carboxylase/oxygenase, small subunit	3e-06	**0.32**	n.s.
FG981225	GDSL-motif lipase	3e-24	**0.40**	n.s.
FG981268	UDP-glucose glucosyltransferase	6e-16	**0.33**	n.s.
Energy				
FG981230	chlorophyll a/b binding protein	6e-103	**0.29**	n.s.
FG981266	chlorophyll a/b binding protein	2e-38	**0.19**	n.s.
FG981217	chlorophyll a/b-binding protein	4e-61	**0.31**	**0.49**
FG981293	chlorophyll a/b-binding protein	3e-38	**0.30**	n.s.
FG981206	chlorophyll a/b-binding protein	3e-08	**0.40**	n.s.
FG981221	chlorophyll a/b-binding protein	1e-38	**0.41**	n.s.
FG981205	chlorophyll a/b-binding protein CP24 precursor	9e-21	**0.34**	n.s.
FG981212	chlorophyll a-b binding protein 3C-like	1e-96	**0.47**	0.55

Accession No.	ANNOTATION	e-value	Leaves x-fold change	Roots x-fold change
FG981211	chlorophyll a-b binding protein 4, chloroplast precursor (LHCII type I CAB-4) (LHCP)	8e-110	**0.37**	0.57
FK265365	light-harvesting chlorophyll a/b-binding protein	4e-32	**0.29**	n.s.
FG981208	light-harvesting chlorophyll a/b-binding protein	2e-52	**0.27**	**0.49**
FG981294	light-harvesting chlorophyll a/b-binding protein	2e-63	**0.29**	**0.17**
FG981231	unnamed protein product (monooxygenase)	8e-51	**2.66**	n.s.
FG981215	chlorophyll A-B binding protein (CAB), putative	6e-120	**0.27**	**0.33**
Transcription				
FG981274	BTB/POZ; MATH	2e-27	**2.53**	n.s.
FG981252	unnamed protein product, DHHC zinc finger domain	2e-12	**0.41**	n.s.
FG981299	RWP-RK domain-containing protein	2e-13	n.s.	**0.50**
FG981285	transcription factor WRKY14	5e-25	n.s.	**0.30**
FG981258	hypothetical protein putative zinc fingers with GTPase activating proteins (GAPs) towards the small GTPase, Arf.	1e-67	**4.95**	**28.17**
Cell rescue, defence and virulence				
FG981200	cationic peroxidase	2e-31	**2.19**	1.69
FG981272	chitinase class II	2e-56	**4.16**	**21.78**
FG981234	dehydrin	4e-06	**10.55**	**33.40**
FG981300	disease resistance-responsive family protein (dirigent-like protein)	2e-06	n.s.	**0.44**
FG981253	heat shock protein 70	6e-74	**0.40**	0.57
FG981279	heat shock protein 70-3	3e-59	**0.33**	**0.28**
FG981245	ascorbate peroxidase	1e-43	n.s.	**2.48**
FG981288	osmotin-like protein	2e-44	n.s.	**0.39**
FG981273	pathogenesis-related protein 10	1e-30	n.s.	**0.50**
FK265367	pathogenesis-related protein 8	4e-48	**5.07**	n.s.
FG981228	pathogenesis-related protein 8	1e-94	n.s.	**6.16**
Cellular communication/signal transduction mechanism				
FG981179	Ca-binding protein	6e-49	**2.59**	**9.56**
FG981229	hypothetical protein (EF-hand, calcium binding motif)	3e-16	**2.65**	n.s.
FG981261	hypothetical protein with Ca-binding motif	3e-16	**2.65**	n.s.
FG981177	leucine-rich repeat protein kinase, putative	7e-10	**3.32**	n.s.
FG981233	PERK1-like protein kinase	2e-13	**4.13**	n.s.
FG981244	putative protein kinase	2e-40	n.s.	**2.01**

Accession No.	ANNOTATION	e-value	Leaves x-fold change	Roots x-fold change
FG981254	putative receptor protein kinase PERK1	2e-66	n.s.	**7.25**
FG981210	receptor protein kinase	1e-66	**0.22**	n.s.
Cellular transport, transport facilitation and transport routes				
FG981181	delta-tonoplast intrinsic protein	7e-100	**0.37**	n.s.
FG981226	MRP-like ABC transporter	4e-33	**0.32**	n.s.
FG981237	synaptobrevin/VAMP-like protein	1e-88	**2.18**	**5.40**
Systemic interaction with the environment				
FG981193	abscisic stress ripening-like protein	8e-09	**2.67**	n.s.
FG981295	GASA, Gibberellin regulated protein	3e-22	0.80	**0.47**
FG981239	putative auxin-repressed protein	1e-14	**2.59**	**6.44**
FG981242	putative NAD(P)-dependent cholesterol dehydrogenase	4e-40	1.93	**2.00**
Protein fate				
FG981238	ubiquitin	4e-41	1.61	**3.24**
FG981259	peptidylprolyl isomerase (cyclophilin)	1e-43	**0.42**	n.s.
FG981220	putative F-Box protein	1e-14	**0.26**	0.57
Protein synthesis				
FG981243	chloroplast-specific ribosomal protein	8e-62	**3.12**	**4.80**
FG981227	putative ribosomal protein S26	2e-23	**0.28**	n.s.
FG981301	60S ribosomal protein L35a	8e-12	**2.56**	n.s.
FK265368	unnamed protein product (Ribosomal protein S8e)	5e-75	**3.11**	**3.37**
FG981291	putative translation factor	2e-13	**2.09**	0.62
Protein with binding function or cofactor requirement				
FG981201	homeodomain protein Hfi22	4e-52	**0.37**	n.s.
FG981219	luminal-binding protein precursor (BiP) (78 kDa glucose-regulated protein homolog) (GRP 78)	2e-11	**0.37**	n.s.
FG981241	metallothionein-like protein type 2	6e-26	**3.35**	**10.95**
FG981224	RAB1C	7e-90	**0.34**	n.s.
FG981176	RNA-binding/nucleic acid binding	2e-49	**18.34**	**32.47**
FG981178	RNA-binding/RNA-direct DNA polymerase	8e-22	**12.23**	**15.64**
FG981264	unknown protein with binding function	6e-23	**0.62**	**0.42**
Cell cycle and DNA processing				
FG981284	H2A histone	2e-26	n.s.	**0.42**

Accession No.	ANNOTATION	e-value	Leaves x-fold change	Roots x-fold change
Cellular signalling				
FG981265	SPX (SYG1/Pho81/XPR1) domain-containing protein	9e-29	n.s.	**2.22**
Development				
FG981223	embryo-abundant protein EMB, putative	5e-27	**0.37**	n.s.
Regulation of metabolism and protein function				
FG981240	PinII-type proteinase inhibitor 11	3e-118	**2.49**	**6.59**
FG981194	putative proteinase inhibitor II	3e-19	1.36	**2.26**
Storage protein				
FG981207	24K germin like protein	7e-47	**0.26**	n.s.
Unknown				
FG981192	hypothetical protein (with similarities to RD 29B)	3e-81	**43.47**	**30.76**
FG981189	hypothetical protein	2e-53	**0.34**	**0.54**
FG981214	no similarities		**0.09**	n.s.
FG981246	no similarities		**4.68**	**18.13**
FG981257	no similarities		**16.31**	**22.87**
FG981267	no similarities		**19.67**	**29.89**
FG981216	no similarities		**0.44**	n.s.
FG981183	no similarities		**2.14**	n.s.
FG981278	no similarities		**0.66**	**0.16**
FG981270	no similarities		**2.66**	**4.92**
FG981188	no similarities		**2.11**	n.s.
FG981283	no similarities		**0.25**	**0.36**
FG981297	no similarities		n.s.	**0.15**
FG981271	no similarities		1.29	**3.52**
FG981218	no similarities		**2.33**	n.s.
FG981174	no similarities		**2.00**	**11.95**
FG981222	no similarities		**0.06**	n.s.
FG981256	no similarities		n.s.	**2.63**
FG981303	no similarities		n.s.	**0.43**
FG981281	no similarities		**12.02**	n.s.
FG981289	no similarities		n.s.	**2.37**
FG981232	no similarities		n.s.	**2.37**
FK265366	no similarities		**2.52**	**n.s.**

Accession No.	ANNOTATION	e-value	Leaves x-fold change	Roots x-fold change
FG981296	unnamed protein product	6e-55	n.s.	**0.45**
FG981269	unnamed protein product	4e-36	**6.36**	n.s.
FG981209	unnamed protein product	2e-18	**2.10**	1.51
FG981302	unnamed protein product	1e-06	**0.27**	n.s.
FG981263	unknown	1e-10	**3.16**	n.s.

Supplement 3

Table S3: ESTs significantly more abundant in different libraries (Tissue: L = leaves, R = roots, Library: For = forward subtracted, most probably up-regulated genes, Rev = reverse subtracted, most probably down-regulated gene. Reliability of the BLAST search is given for each clone by the e value.)

Accession No.	Annotation	Tissue	Library	e-value
Metabolism				
FG981186	enoyl-CoA hydratase/isomerase family protein	L	For	2e-17
FG981196	unnamed protein product (aminotransferase class I and II)	L	For	4e-70
FG981235	transketolase	R	For	2e-37
FG981262	cytochrom P450 like_TBP	R	For	2e-51
FG981290	pantoate-beta-alanine ligase	R	Rev	2e-78
Transcription				
FG981203	unnamed protein product; Zinc finger in Ran-binding protein and others (zf-RanBP)	L	For	3e-59
FG981249	unnamed protein product; uncharacterised conserved protein, contains RING Zn-finger	R	For	5e-22
FG981255	unnamed protein product, SWIB/MDM2 domain	R	For	3e-39
Cell rescue, defence and virulence				
FG981251	heat shock protein	R	For	7e-77
FG981280	unnamed protein product (harpin-induced protein 1 (Hin1))	R	Rev	1e-38
Cellular communication/signal transduction mechanisms				
FG981202	ADK/ATPADK1 (adenosine kinase); nucleotide kinase	L	For	6e-81

Accession No.	Annotation	Tissue	Library	e-value
Cellular transport, transport facilitation and transport routes				
FG981247	unnamed protein product preprotein translocase subunit SecY [Intracellular trafficking and secretion]	R	For	1e-125
Systemic interaction with the environment				
FG981175	DELLA protein	L	For	2e-15
Protein fate				
FG981286	proteasome inhibitor-related protein	R	Rev	3e-22
Protein synthesis				
FG981184	60S ribosomal protein L2	L	For	5e-37
Protein with binding function or cofactor requirement				
FG981190	ARPC1b (actin-related protein C1b); nucleotide binding	L	For	6e-45
FG981197	CND41, chloroplast nucleoid DNA binding protein	L	For	6e-43
FG981298	GRF6 (GF14 LAMBDA); protein phosphorylated amino acid binding	R	Rev	2e-73
Unknown				
FG981182	unnamed protein product	L	For	2e-21
FG981236	unnamed protein product	R	For	3e-29
FG981248	no similarities	R	For	
FG981250	no similarities	R	For	

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

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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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

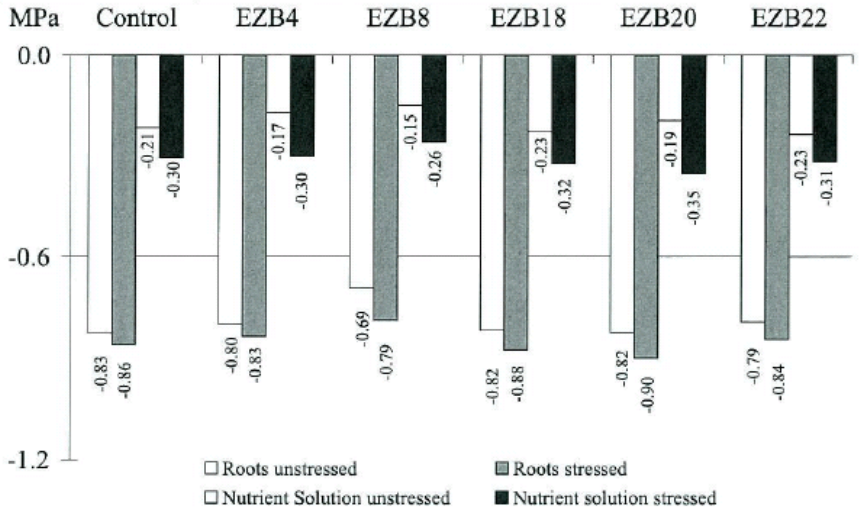
The selected genes, their corresponding accession numbers, and primers used are listed in Table 2. We tested a *Capsicum annuum* nonspecific lipid transfer protein gene (*CaLTPI*) (Jung et al. 2003), a *Capsicum annuum* SAR8.2 gene (*CaSAR82A*) (Lee and Hwang 2003), a *Capsicum annuum* ACC oxidase gene (*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 µmol·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).

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 ($P < 0.05$).

Results and discussion

Biomass

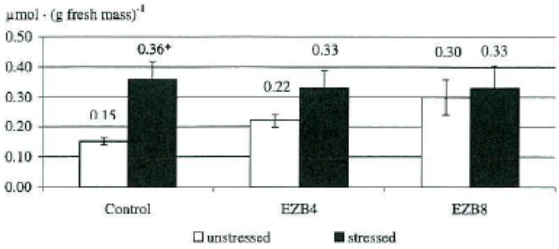
Endophytic bacteria were isolated from pepper plants (Frank Rasche, unpublished data) and analyzed for ACC deaminase production. Five ZB endophytes belonging to the genera *Arthrobacter*, *Bacillus*, and *Microbacterium*, which showed ACC deaminase activity but, which according to their identity, did 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.

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.

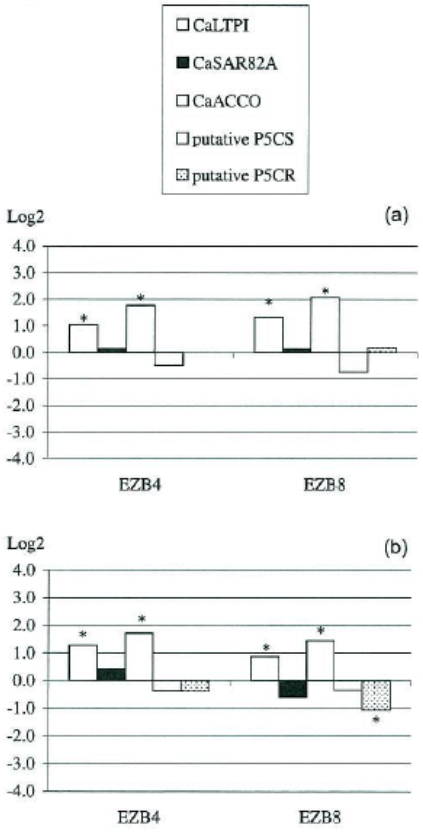
The proline level in leaves of noninoculated plants increased significantly owing to PEG treatment (Fig. 2). Inoculated stressed plants also accumulated the osmolyte proline to nearly the same level. However, the proline concentration was higher in leaves of unstressed plants inoculated with strains 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.

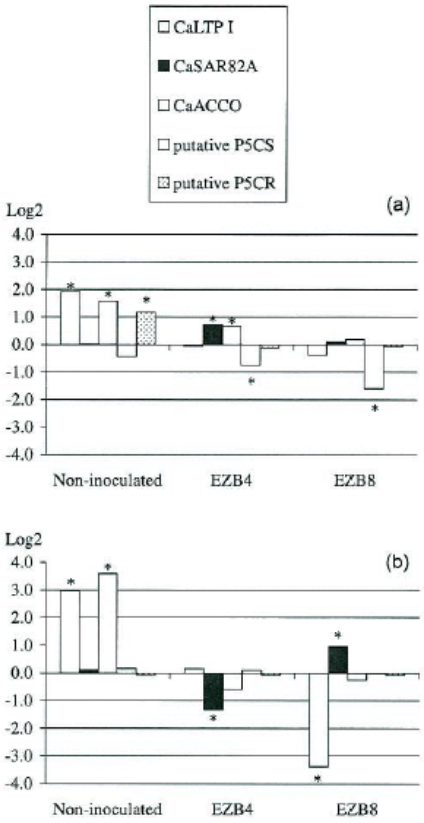
CaLTPI is the second gene that was significantly upregulated in leaves and roots of noninoculated plants because of mild osmotic stress (Figs. 4*a* and 4b). Inoculation with strain EZB4 resulted in a stable gene expression in leaves and roots. In contrast, a significant downregulation was observed in 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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2.3. Results of the analysis of a multi-species micro-array

The following paper is under discussion and several experiments are still ongoing.

Assembly of a Multi-species Drought Stress Microarray

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Number of tables: 7

Number of figures: 1

Abstract:

One approach to genomic research in plants without extant microarray platform is the use of cross-species hybridisations. To test the ability of a stress-specific multi-species microarray an array with putative drought responsive genes of six different plant species was assembled. Pepper, potato, poplar, sweet potato, wheat and pine probes derived from the platform for integrated clone management (PICME) clone depository (http://www.picme.at). Hybridisation was done with cDNA of mild drought stressed pepper plants. Hybridisation rates ranged from 57.9 % (pine probes) to 95.7% (potato probes). Analysis of gene expression changes measured for an assortment of homologous genes and pathways revealed a certain loss of power. Nevertheless and despite the use of distantly related species for all evaluated pathways and gene families several non-target probes gave a feasible result. Non of the measured expression rate was in clear contrast to the performance of the pepper probes.

Keywords: multi-species microarray, drought, pepper

Introduction

Drought is one of the most important factors limiting growth and development of plants. Therefore improved drought-tolerance is a major objective of many breeding programs in different crop species.

Plants respond to water deficit with a series of common physiological, cellular and molecular processes. Many drought inducible genes have already been identified by molecular and genomic analysis in *Arabidopsis* (Seki et al. 2001, Seki et al. 2002), rice (Rabbani et al. 2003), tomato (Ouyang et al. 2007), potato (Rensink et al. 2005) and many other plants. DNA microarrays make it possible to monitor the expression levels of a great number of genes simultaneously (Bowtell, 1999; Eisen and Brown 1999). Thus microarrays can be used to investigate the mechanisms of biological processes, to group genes into functional pathways or to assign functions to previously un-annotated genes (Stoughton 2005).

Currently DNA arrays are available for a number of species, among these Arabidopsis, citrus, cotton, maize, alfalfa, poplar, rice soybean, sugar cane, tomato, grape and wheat. Nevertheless for many species standard microarray platforms are not available. Cross-species array may be a possibility to overcome this problem. This technology has already been used to analyse gene expression differences within and between closely related species. For example tomato microarrays were used to analyse gene expression changes in pepper and eggplant (Moore et al. 2005), expressions of wheat genes were studied on a whole genome rice chip platform (Xu et al. 2008) or an Arabidopsis array was used for transcriptional profiling of oilseed rape seeds (Girke et al. 2000). Not only in plants but also in animals cross-species array has been used. In a similar manner gene expression levels of *Drosophila simulans* were analysed using a *D. melanogaster* microarray (Rifkin et al. 2003), or human microarrays were used to study expression patterns of different tissues of rhesus macaques (Vahey et al. 2003) or chimpanzee (Bigger et al. 2001).

To examine the ability of a stress-specific multi-species array we assembled a microarray with putative drought responsive genes of six different plant species, namely pepper, potato, wheat, sweet potato, poplar and pine. All ESTs used were available at the PICME clone depository at the Austrian Institute for Technology (http://www.picme.at).

The impact of the phylogenetic distance between probe and target on the quality of the microarray was estimated by determination of the hybridisation rates and assessment of the reliability of the gene expression changes of homologous genes.

Material and methods

Literature research revealed more than 1000 interesting drought stress related genes. DNA sequences of these genes served as template for database searches. After BLAST alignment corresponding ESTs of poplar (*Populus euphratica*), sweet potato (*Ipomoea batatas*), pine (*Pinus pinaster*), wheat (*Triticum aestivum*) and potato (*Solanum tuberosum*) available at the PICME clone depository (www.picme.at) were selected. In addition pepper ESTs of an SSH-library of early drought stressed pepper plants as well as several housekeeping genes of pepper (actin, α-tubulin, β-tubulin, cyclophilin, 18S rRNA, RuBisCo, GAPDH, transcription initial factor, elongation factor) were added as positive controls. As negative controls cDNA of human genes were included in multiple copies in an ordered distribution across the microarray.

The slides were hybridised with labelled cDNA of leaves and roots of drought stressed pepper plants. The plants were harvested upon full development of 6 to 7 leaves and after withholding water for one week. Four microarrays each were hybridised with labelled cDNA of leaves and roots of drought stressed and control pepper plants of four different biological replicates. Half of the slides were used as dye swap with reciprocal labelled cDNAs. Preparation of the slides, RNA isolation and transcription, labelling of cDNA and hybridisation at 42°C was performed as previously described (Sziderics et al. 2008).

The slides were scanned with an LS Reloaded scanner (Tecan) and the resulting images were processed with GenPixPro 6.0 software (Axon Instruments Inc.). Hybridisation rate per species was calculated as percentage of spots showing a clear signal. For determination of gene expression changes a spatial and intensity-dependent (LOWESS) normalisation method was employed to normalise the ratio values of the raw data, which were afterwards statistically analysed using LIMMA software package in R (Smyth 2004) from the Bioconductor project (http://www.bioconductor.org). The cut off value was set to $p < 0.05$.

Results and discussion

BLAST alignment of in literature found drought related genes resulted in a multi-species microarray with probes of poplar (440), sweet potato (47), pine (76), wheat (29) and potato (52). As control 3231 ESTs of a pepper SSH library, several housekeeping genes and human probes were added. Table 1 gives a rough overview on the phylogenetic distance of the species used.

Tab. 1: Simplified phylogenetic systematics of the species used on the array

		Order	**Family / Tribe**		**Genus**
Coniferophyta	Coniferopsida	Coniferales	Pinaceae		Pinus
Magnolio-phyta	Mono-cotyledons	Poales	Poaeceae	Triticeae	Triticum
	Eudi-cotyledons	Malpighiales	Salicaceae	Saliceae	Populus
		Solanales	Convulvulaceae	Ipomoeeae	Ipomoea
			Solanaceae	Solaneae	Solanum
				Capsiceae	Capsicum

First analysis of the microarrays was the calculation of the hybridisation rate of labelled pepper cDNA to the probes of different species by counting the number of hybridised probes. The reproducibility of the results within the four replicates was very high and negative controls gave no signal. The results are summarised in table 2.

Tab. 2: % hybridised probes of the multi-species stress-array after hybridisation with labelled cDNA of pepper leaves and roots, n = 4; number of hybridised probes ± deviation out of probes of a certain species)

	Leaves	Roots
Potato (*Solanum tiberosum*)	95.7 % (50 ± 1 / 52)	87.0 % (45 ± 2 / 52)
Wheat (*Triticum aestivum*)	75.9 % (22 ±1 / 29)	50.9 % (15 ± 3 / 29)
Sweet potato (*Ipomoea batatas*)	71.3 % (34 ± 1 / 47)	46.3 % (22 ± 4 / 45)
Poplar (*Populus euphratica*)	64.7 % (285 ± 15 / 440)	38.0 % (167 ± 40 / 440)
Pine (*Pinus pinaster*)	57.9 % (44 ± 1 / 76)	34.5 % (26 ± 6 / 45)
Pepper (*Capsicum annuum*)	40.0 % (1280 / 3251)	28.0 % (907 / 3251)

The highest hybridisation rate was achieved with probes of potato, which is the closest related species. Interestingly hybridisation with wheat was better than with poplar which is in contrast to the phylogenetic distance. This can be assigned to the different habit as a tree and herbaceous plant. Unfortunately these results cannot be compared with the hybridisation rates of pepper, which were very low. This can be assigned to the pepper probes, which were derived of an SSH library. This is a technique to enrich for differentially expressed genes and genes with a low abundance (Diatchenko et al., 1996). These low abundance mRNAs are more difficult to examine using microarrays than high abundance mRNAs due to the effect of concentration on hybridisation kinetics and signal-to-noise ratios (Rondeau et al., 2005).

The method of simply counting the number of hybridisations to analyse cross-species arrays has been used before in other (Tsoi et al. 2003, Chismar et al. 2002). However, this method gives only an indication on the possibility of a hybridisation, but does not reflect the quality of the microarray. It gives no information on hybridisation specificity or reproducibility of gene expression results (Bar-Or et al. 2007).

It is well known, that closely related species show a good hybridisation and cross species hybridisations are widely performed. For example Becher et al. (2004) used *Arabidopsis thaliana* GeneChips to analyse the transcriptomes of *A. halleri*, Xu et al. (2008) investigated the wheat transcriptional profile with a rice chip and Moore et al. (2005) compared gene expressions of tomato, pepper and eggplant using a tomato microarray. In these studies it is assumed, that a microarray designed for one species can be used to determine differential expression in another species without substantial loss of information. However, a possible problem of cross-species microarrays are sequence mismatches. This is especially important for oligo arrays, whereas cDNA microarrays are assumed to be more suitable for cross-species hybridisations as the larger probes might reduce the effects of interspecies differences on the results (Bar-Or et al. 2006).

Anyway, the quality of a microarray depends on the reproducibility of gene expressions differences. In tables 3 to 7 examples of measured gene expression differences after cross hybridisation of poplar, sweet potato, pine and potato probes are compared with the results obtained with ESTs of the pepper library for an assortment of pathways and homologous genes. In all cases few cross-species hybridisations resulted in a similar gene expression as obtained with pepper probes. Nevertheless, many cross-species probes gave not significant signals or no signal at all.

With regard to photosynthesis 12 different down-regulated chlorophyll a/b-binding proteins were isolated by SSH and 17 homologous ESTs of poplar, sweet potato and pine were available at the PICME clone depository. One poplar EST gave a signal similar to the pepper ESTs. Another EST of sweet potato also showed down-regulation, but to a lower extent. All other homologue ESTs showed no differential expression or did not result in an evaluable signal (Table 3). Reduced photosynthesis and down-scaling of the photosynthetic carbon and energy metabolism under water deficit is well documented and is reflected in down-regulation of genes encoding photosynthesis associated proteins such as chlorophyll binding and photosystem related proteins (Bray, 2002). Also the abundance of the RuBisCo small subunit is known to be reduced under drought (Reddy et al., 2004). In accordance to this probes of sweet potato and poplar related to photosystem I and II as well as a RuBisCo

small subunit homologue of poplar showed down-regulation (Table 3).

Ascorbate peroxidases are an example of a multi-gene family which encodes functionally diverse isoforms localised in different subcellular compartments (Shigoka et al. 2002, Teixeira et al. 2006). By SSH an EST encoding an ascorbate peroxidase highly induced in roots was isolated. In accordance with this some homologue probes of poplar and sweet potato also showed up-regulation (Table 4). The up-regulation of the cross-species probes in leaves, which was not seen with the pepper probe, may be assigned to different isoforms and is a well known effect under drought stress (Reddy et al. 2004, Bray 2002). Nevertheless, six probes gave no significant signal.

Another multi-gene family is represented by heat shock proteins (HSP). Two pepper clones encoding 70 kDa HS proteins were significantly repressed. Also two poplar probes showed down-regulation, but to a much lower extent. Again several probes resulted in not significant or no signals.

An example of a well known pathway induced by drought stress it the synthesis of proline via glutamate (Fig. 1) (Hare et al. 1999, Kavi Kishor et al. 2005).

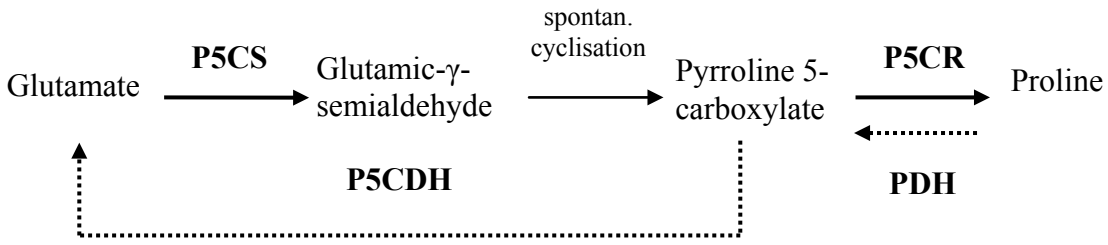

Fig. 1: Simplified proline pathway via glutamate

Both putative proline synthesis related ESTs of pepper were slightly up-regulated under moderate drought stress. A poplar probe of P5CS gave no significant gene expression difference, whereas a poplar probe encoding P5CDH showed a significant down-regulation and a poplar probe annotated as proline transporter revealed a clear up-regulation (Table 6). This complies with proline metabolism and the well known accumulation of proline under drought stress (Kavi Kishor et al. 2005).

Hybridisation of potato and pepper probes encoding chitinase class II revealed up-regulation in leaves and roots (Table 7). However, the measured gene expression was smaller for the potato probes.

In summary for all mentioned pathways and gene families several non-target probes gave a feasible result and non of the measured expression rate was in clear contrast to the performance of the pepper probes. This is especially notable as the microarray contained probes of distantly related species. Cross-species arrays have already been widely used for closely related species assuming that a microarray designed for one species can be used to analyse differential expression in another species without substantial loss of information. However, by studying gene expressions in potatoes using either potato or tomato custom microarrays Bar-Or et al. (2006) found inconsistent results and thus suggest that transcriptomic data of cross-species hybridisations need to be critically analysed to reflect biological processes. The problem is the extremely variable degree of homology between probes. In the presence of sequence mismatches, relative hybridisation intensity will reflect both differences in transcript abundance levels as well as differences in hybridisation kinetics (Gilad et al. 2005).

In this preliminary test a high number of non-target probes hybridised, but showed no gene expression change. This may reflect a certain loss of power, which may be assigned to a reduction of transcripts that hybridise to the particular spot caused by sequence mismatching. Thus mismatching results in a reduced hybridisation signal. A reduced signal has been suggested in many cross-species studies (Moore et al. 2005, Gilad et al. 2005, Bar-Or et al. 2006). As a consequence the number of detected significant genes is reduced, if these are identified by standard statistical tests (Bar-Or et al. 2007).

In conclusion it was demonstrated that cross-hybridisation occurs even with distantly related probes. Nevertheless compared with species-specific arrays this multi-species array exhibits a remarkable loss of power. Thus small gene expression changes can hardly be detected. In addition due to sequence mismatches a critical analysis of the results is need.

Tab. 3: Measured expressions of photosynthesis related genes in leaves of drought stressed pepper plants after cross-hybridisation to probes of different plant species; $p < 0.01$; n.s. = not significant; pepper: mean gene expression level of 12 different chlorophyll a/b binding proteins isolated by SSH; - = no or very week signal after hybridisation

Species	Annotation	Log2 ratio
Pepper	Chlorophyll a/b binding protein	-1.80
Populus	Chlorophyll a/b binding protein 13 chloroplast	-1.76
Sweet potato	Chlorophyll a/b binding protein (Lhcb6 protein)	-0.54
Sweet potato	Chlorophyll a/b binding protein (Lhcbl-1)	n.s.
Sweet potato	Chlorophyll a/b binding protein precursor (Cab 27)	n.s.
Sweet potato	Chlorophyll a/b binding protein	n.s.
Populus	Chlorophyll a/b binding protein 151 chloroplast precursor (LHCII type II CAB-151)	n.s.
Populus	Chlorophyll a/b binding protein, LHCII type III	n.s.
Populus	Chlorophyll a/b binding protein CP26	n.s.
Populus	Chlorophyll a/b-binding protein (cab-11)	n.s.
Populus	Chlorophyll a/b-binding protein CP24	n.s.
Populus	Chlorophyll a/b binding protein 3 chloroplast	-
Populus	Chlorophyll a/b-binding protein type I	-
Populus	Chlorophyll a/b binding protein precursor	-
Populus	Chlorophyll a/b binding protein precursor	-
Sweet potato	Chlorophyll a/b binding protein LHCII type III	-
Sweet potato	Chlorophyll a/b binding protein LHCII type III	-
Pinus	Chlorophyll a/b binding protein	-
Sweet potato	Photosystem I reaction centre subunit psaN precursor	-2.43
Sweet potato	Photosystem I subunit XI precursor	-1.83
Populus	Photosystem I psaH protein	-1.76
Populus	Photosystem I subunit VI precursor	-1.27
Sweet potato	Photosystem I psaH protein	-1.03
Potato	Photosystem I-H precursor homolog	-0.43
Populus	Photosystem I reaction centre subunit XI chloroplast precursor (PSI-L)	-
Populus	Photosystem I-N subunit	-
Sweet potato	Photosystem II 22Kda precursor protein (psbS)	-
Pinus	Photosystem II 22 KDA	-
Pepper	Ribulose 1,5 bisphosphate carboxylase/oxygenase, small subunit	-1.65
Populus	Ribulose bisphosphate carboxylase small chain f1	-0.88

Tab. 4: Measured expression rates of genes encoding ascorbate peroxidase in leaves and roots of drought stressed pepper plants after cross-hybridisation to probes of different species; p< 0.01; n.s. = not significant; - = no or very week signal after hybridisation

Species	Annotation	Log2 ratio	
		Leaves	Roots
Pepper	Ascorbate peroxidase	n.s.	1.31
Poplar	Ascorbate peroxidase	1.42	1.54
Potato	Ascorbate peroxidase	n.s.	n.s.
Sweet potato	Cytosolic ascorbate peroxidase	1.78	-
Poplar	Ascorbate peroxidase	n.s.	n.s.
Poplar	L-ascorbate peroxidase	n.s.	0.49
Poplar	L-ascorbate peroxidase	n.s.	n.s.
Poplar	L-ascorbate peroxidase	n.s.	n.s.
Poplar	L-ascorbate peroxidase	n.s.	-
Poplar	L-ascorbate peroxidase	n.s.	n.s.
Pinus	L-sacorbate peroxidase	n.s.	n.s.

Tab. 5: Measured expressions of HSP 70 in leaves and roots of drought stressed pepper plants after cross-hybridisation to probes of different species; p< 0.01; n.s. = not significant; - = no or very week signal after hybridisation

Species	Annotation	Log2 ratio	
		Leaves	Roots
Pepper	Heat shock protein 70	-1.31	-1.00
Pepper	Heat shock protein 70-3	-2.00	-2.12
Poplar	Heat shock cognate 70 kDa protein 1 (Hsc70.1)	-0.47	-0.76
Poplar	Heat shock cognate 70 kDa protein	-0.59	-
Poplar	Heat shock cognate 70 kDa protein	n.s.	n.s.
Poplar	Heat shock 70 kDa protein	n.s.	-
Poplar	Heat shock protein 70 cognate	n.s.	-
Poplar	Heat shock protein 70	-	-
Pinus	Heat shock protein 70 kDa protein	-	-

Tab. 6: Measured expressions of proline related genes in leaves and roots of drought stressed pepper plants after cross-hybridisation to probes of different species; $p < 0.01$; n.s. = not significant

Species	Annotation	Log2 ratio	
		Leaves	Roots
Pepper	Putative Delta 1-pyrroline-5-carboxylate synthetase (P5CS)	0.49	0.68
Poplar	Delta 1-pyrroline-5-carboxylate synthetase (P5CS)	n.s.	n.s.
Pepper	Putative Delta 1-pyrroline-5-carboxylate reductase (P5CR)	0.58	0.59
Pepper	proline oxidase/dehydrogenase 2	-0.74	-1.84
Poplar	Delta-1-pyrroline-5-carboxylate dehydrogenase (P5CDH)	-0.54	-0.66
Poplar	proline transporter 2	1.10	1.80

Tab. 7: Measured expressions of different genes encoding chitinase class II in leaves and roots of drought stressed pepper plants after cross-hybridisation to potato probes; $p < 0.01$; n.s. = not significant

Species	Annotation	Log2 ratio	
		Leaves	Roots
Pepper	Chitinase class II	2.06	4.44
Potato	Chitinase class II	1.77	3.15

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3. CONCLUSION

Drought stress is one of the major limitations to crop yield in pepper (González-Dugo et al. 2007). To develop plants with enhanced tolerance to water deficit a fundamental knowledge of the physiological, biochemical and molecular networks is essential. Furthermore it is known, that endophytic bacteria can enhance drought and osmotic stress resistance.

In this thesis it could be shown, that pepper plants had already responded to increasing water deficit by accumulation of various metabolites, when first wilting symptoms were observed, but a change in leave water content was not yet measurable. The response to early drought stress differed clearly between roots and leaves. Whereas in roots raffinose and glucose increased, fructose and sucrose were accumulated in leaves. The concentrations of spermine, spermidine, putrescine and cadaverine also increased in leaves, whereas the content of these polyamines was reduced in roots. An explanation may be the fact, that polyamines induce stomata closure and strongly inhibit their opening (Liu K. et al., 2000). Galactinol and hydroxyproline were accumulated in both plant organs upon drought stress. In contrast the content of proline increased tremendously in roots but was reduced in leaves. Proline was also accumulated in leaves of pepper plants inoculated with endophytic bacteria. This confirms that this key metabolite is synthesised in a wide range of abiotic and biotic stresses (Hare and Cress, 1997).

Besides biochemical analysis the gene expression of drought stressed as well as inoculated pepper plants was analysed. About 3200 ESTs of a pepper SSH library and additionally 644 putative drought responsive ESTs of poplar, sweet potato, pine, wheat and potato were analysed in a multi-species microarray. Hybridisation of this preliminary multi-species microarray with cDNA of drought stressed pepper plants revealed a hybridisation rate ranging from 57.9% (pine probes) to 95.7% (potato probes) depending on the plant species and the phylogenetic distance to pepper. Analysis of gene expression changes measured for an assortment of homologous genes demonstrated a certain loss of power. However, none of the measured expression rate was in clear contrast to the results of the pepper probes.

This simultaneous analyses of transcriptional and metabolic profiles of leaves and roots revealed a match between the expression of proline synthesis related genes and the accumulation upon water deficit. Genes encoding for pyrroline 5-carboxylate synthetase (P5CS) and pyrroline 5-carboxylate reductase (P5CR) were up-regulated in roots and leaves. In accordance a pepper EST encoding for the proline dehydrogenase and a poplar EST

encoding for pyrroline 5-carboxylate dehydrogenase were down-regulated. The reduced concentration of proline in leaves suggests a transfer into roots. Within the SSH-library no pepper EST encoding for a proline transporter was found, but a poplar probe annotated as a proline transporter revealed clear up-regulation. Due to inoculation of the pepper plants with endophytic bacteria the gene expression patterns of P5CS and P5CR during moderate osmotic stress were altered depending on the bacterium applied.

The evaluation of the microarrays resulted in 109 unique drought responsive ESTs. Their expression differed within the analysed plant organs. Only 18 ESTs were significantly up-regulated and 9 down-regulated in both leaves and roots. Not a single EST was regulated into opposite direction within the two organs. The large number of probes of the SSH-library (> 60%), which failed to be detected by microarray analysis, can be ascribed to the isolation of low abundance mRNAs. SSH is a technique especially designed for the detection of rare transcripts (Diatchenko et al., 1996), which are difficult to examine due to the effect of concentration on hybridisation kinetics and signal-to-noise (Rondeau et al., 2005). This assumption could be verified by qRT-PCR using a transcriptase with an enhancer. Expression of five ESTs isolated by forward subtraction from leaves were shown to be clearly up-regulated due to drought. By hybridisation of the microarray with subtracted cDNA additional 286 ESTs were assigned to certain libraries, which gives an indication on their different expression.

The sequenced ESTs could be assigned to a broad spectrum of functional classes, which reflects the complexity of the physiological response of the plants. A great number of ESTs is involved in metabolic pathways. One of these ESTs up-regulated in leaves of drought stressed plants encoded for 1-aminocyclopropane-1-carboxylate oxidase (ACC oxidase), which catalysis the last step of ethylene biosynthesis (Bleecker and Kende, 2000). The plant hormone ethylene acts as a second messenger within a generic signal transduction pathway under abiotic and abiotic stress conditions (Liu et al., 2000). Inoculation of osmotically stressed pepper plants with two ACC-deaminase producers resulted in a clearly reduced up-regulation or even down-regulation of the stress inducible gene *CaACCO* as compared to gene expression in non-inoculated plants. Thus it can be concluded that these endophytes induced abiotic stress relief via ACC deaminase activity.

A large group of the sequenced ESTs of the SSH library most probably is involved in energy metabolism, which included several down-regulated chlorophyll a/b-binding and light harvesting proteins. Also a number of cell rescue related ESTs including a highly up-regulated dehydrin, peroxidases and heat shock proteins were isolated. Further genes were

assigned to the categories cellular communication and signal transduction. For more than one quarter of the sequenced ESTs no similarity to known proteins was found. Among these six ESTs were more than ten-fold up-regulated. This indicates, that still a lot of unknown pathways need to be analysed to understand the early response to water deficit.

4. POSTER PRESENTATIONS

1. Sziderics A.H., Wilhelm E., Fluch S. 2004. The effect of abiotic stresses and endophytes on gene expression in pepper (*Capsicum annuum* L.). Poster competition, Seibersdorf, 02/2005

2. Sziderics A.H., Fluch S., Berenyi M., Rasche F., Sessitsch A., Wilhelm E. 2005. The Effects of Abiotic Stress and Endophytes on Gene Expression in Pepper. 17th International Botanical Congress, Vienna, 18. – 23.07.2005

The Effect of Abiotic Stresses and Endophytes on Gene Expression in Pepper (*Capsicum annuum* L.)

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ARC Seibersdorf research G.m.b.H., Biogenetics and Natural Resources, Biotechnology

Abstract:

Water deficit, cold or heat are common environmental conditions that limit crop production. Within the next 25 years a world wide reduction of cultivated area by 30% due to water deficit or salinity is predicted thus leading to enhanced environmental stress. Therefore one of the goals of plant breeding is an increased abiotic stress tolerance. Knowledge on the plants' response to stress, including genes involved as well as alterations in signalling pathways are necessary for a holistic understanding of plant resistance to stress leading to improved breeding techniques.

This study aims at creating a stress-array with homologous and heterologous cDNA probes obtained from several plant species. On the one hand we will use a SSH-library of drought-stressed and unstressed plants to reveal pepper ESTs. On the other hand we will select ESTs for candidate genes from already existing libraries of different plant species based on literature research.

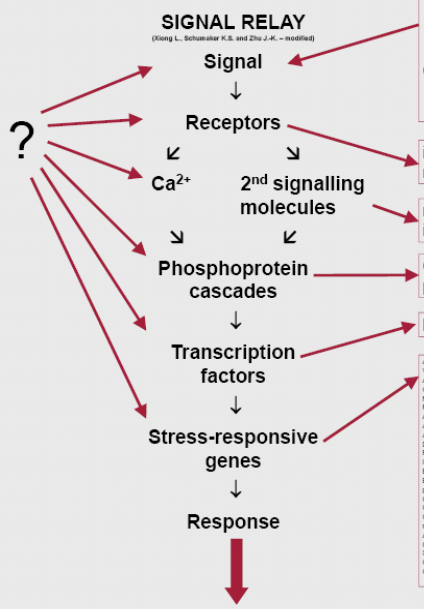
Literature research revealed more than 300 interesting drought-stress related genes of different plant species (e.g. dehydrins, heat shock proteins, lipid transfer proteins, late embryogenesis abundant proteins, ABA-regulated genes etc.). The DNA sequences of these candidate genes serve template for database searches. After BLAST alignment we can select corresponding ESTs of Populus, Ipomoea, Quercus, Triticum etc. available in the PICME-clone depository (www.picme.at) for the production of a generic stress-array. Clones of differentially expressed genes of pepper isolated by SSH will serve as a control on the chip. mRNA of a stress-tolerant (Ziegenhorn Bello) and a stress-sensitive (Milder Spiral) pepper variety will be used to evaluate the stress-array. To correlate gene expressions with the physiological status of stressed and unstressed plants, selected parameters will be measured. In addition gene expression and the influence of non-pathogenic bacteria on stress-behaviour of these two pepper varieties will be tested.

Isolated Endophytes:

Non-pathogenic plant associated bacteria were isolated from pepper (var. Ziegenhorn Bello) and identified by Frank Rasche.

The effects of 5 isolates belonging to the devisions *Actinobacteria* and *Fermicutes* on drought-stressed and unstressed pepper plants are preliminary tested for further decision.

Fig. 1: inoculated pepper plants



Physiological Measurements

To correlate gene expression with the physiological status of stressed and unstressed plants following parameters will be measured:

osmotic potential of roots, leaves and nutrient solutions, content of abscisic acid and proline, activity of superoxide dismutase and peroxidase

Accumulation of:	
2nd signalling molecules	abscisic acid, ethylene, reactive oxygen species, etc.
osmolytes	proline, glycine beatine, mannitol, soluble sugars, polyols etc.
ROS detoxifying enzymes	superoxide dismutase, catalase, glutathion peroxidase, etc.
ROS detoxifying antioxidants	ascorbate, glutathion, tocopherols etc.
water channel proteins	aquaporins, transporters etc.
protecting proteins	LEA proteins, dehydrins, heat shock proteins etc.

Fig. 2: Micro-array

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The Effect of Abiotic Stresses and Endophytes on Gene Expression in Pepper (*Capsicum annuum* L.)

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Abstract:

Endophytes are non-pathogenic plant associated bacteria, that can play important roles in plant vitality and may confer resistance to abiotic or biotic stress. In this study we test the effect of isolates of pepper plants on plant growth and their influence on gene expression change in drought stressed pepper plants. Another aim of this study is the assembly of a cross species stress micro-array. On the one hand we select ESTs for candidate genes from the PICME-clone depository (www.picme.at) of different plant species based on literature research. On the other hand we will use a SSH-library of drought-stressed and unstressed pepper plants to reveal pepper ESTs. This micro-array will be used to tests gene expression of inoculated pepper plants under various conditions. At the same time these pepper plants will be used to evaluate the micro-array.

Effects of Endophytes in Pepper Plants

Plant Growth

Non-pathogenic plant associated bacteria were isolated from pepper var. Ziegenhorn Bello. Pepper plants growing in Magenta Boxes were inoculated by adding 10 ml MS medium containing 10^6-10^7 CFU of endophytes. Four isolates out of five significantly increased growth of the variety Ziegenhorn Bello. Plants of the variety Milder Spiral were very inhomogeneous and therefore no significant effects could be measured (Fig. 1 and 2).

Fig. 1

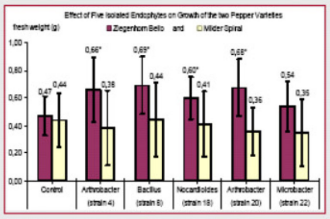

Fig. 2: * = significant increased growth

Drought Stress – Osmotic Pressure

Inoculated pepper plants (Ziegenhorn Bello) were drought stressed for three days by adding 10 ml of MS-medium containing 2 % sugar and ca. 40 % PEG 6000 to the nutrient solutions. The osmotic potential of the nutrient solution and consequently of the roots was slightly increased by the added PEG at an average of -0,10 MPa and -0,05 MPa respectively (Fig. 3).

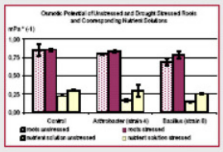

Fig. 3

Effect on Gene Expression

The gene expression changes in roots and leaves of LTP I, ACCO and SAR8.2A were determined by real time PCR with Polyubiquitin as house-keeping gene. LTP I, which encodes a non-specific lipid transfer protein, and SAR8.2A, which is a precursor to a systemic aquired resistance gene, are known to be induced due to drought stress. ACCO encodes ACC oxidase, which catalyzes the production of ethylene. LTP I and ACCO were highly induced due to drought stress in control plants, where plants inoculated with endophytes showed reduced up-regulation or even down-regulation. Regarding SAR8.2A only minor gene expression changes could be measured (Fig. 4 and 5).

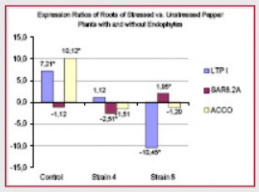

Fig. 4: * = statistically significant

Fig. 5: * = statistically significant

Assembly of a Cross Species Stress-Micro-Array

Literature Rersearch

Literature research regarding stress responsive genes revealed more than 900 interesting genes of different plants e.g. Arabidopsis, tomato, potato, wheat, rice, corn etc.. These genes encoded e.g. dehydrins, heat shock proteins, lipid transfer proteins, late embryogenesis abundant proteins, transcription factors, protein kinases and many unknown proteins.

Alignment

The DNA sequences selected by literature research serve template for database searches. After BLAST alignment we selected corresponding ESTs of Populus, Ipomoea, Quercus, Triticum etc. available in the PICME-clone depository (www.picme.at) for the production of a generic stress-array (Fig. 6).

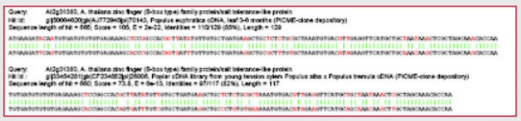

Fig. 6

SSH-library of drought stressed pepper plants

Pepper seeds will be sterilized and put on MS Agar. At the 2-leaf-stage they will be plant into a big box with liquid MS-medium containing 2% sugar (Fig. 7 and 8). Four weeks later half of the plants will serve as unstressed control. The other plants will be drought stressed for 6 or 12 hours by adding PEG 6000 to the nutrient solution. ESTs of differentially expressed genes will be selected using subtractive hybridization technique.

Fig. 7

Fig. 8

Ongoing Tests

We will evaluate the hybridization conditions of this cross-species micro-array using stressed pepper plants. At the same time we will use this micro-array to investigate the influence of endophytes on gene expression of pepper plants Fig. 9).

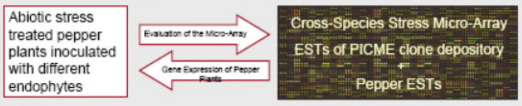

Fig. 9

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