A Metabolic Signature of the Beneficial Interaction of the Endophyte *Paenibacillus* sp. Isolate and In Vitro–Grown Poplar Plants Revealed by Metabolomics

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Submitted 18 February 2009. Accepted 28 April 2009.

Metabolic profiling via gas chromatography coupled to mass spectrometry was used to investigate the influence of endophytic bacteria on shoots of in vitro-grown poplar plants free from culturable endophytic bacteria. The results demonstrate that the occurrence of an endophytic *Paenibacillus* strain strongly affects the composition of the plant metabolites of in vitro-grown poplars. Eleven metabolites were significantly changed between inoculated and non-inoculated poplar plants as determined by two independent experiments. Detected shifts in the primary metabolism of the poplar plants pointed to a mutualistic interaction between bacteria able to fix nitrogen and the host plant with altered nitrogen assimilation patterns. The corresponding metabolic signature comprises increased asparagine and urea levels as well as depleted sugars and organic acids of the tricarboxylic acid cycle. These observations coincide with the fact that the *Paenibacillus* sp. strain P22 is able to grow without nitrogen in the medium, indicating nitrogen fixation from the air also known from other *Paenibacillus* spp. In combination with the detected plant-growth-promoting effects of the endophyte *Paenibacillus* P22, a novel mutualistic interaction is observed.

The presence of bacteria, introduced in cultures as endophytes or later during culture handling, is frequently observed in tissue cultures of trees. Endophytic bacteria sometimes remain covert or latent with no obvious growth on tissue culture medium (Ewald et al. 2000) but they can also reduce growth rate, retard rooting, and even cause plant death (Leifert and Waites 1992). On the other hand, several bacterial endophytes have been reported to support growth and increase the vitality of plants (Frommel et al. 1991; Glick et al. 1998; Lambert and Joos 1989). Recently, we found high densities of *Paenibacillus* spp. in tissue cultures from different woody plants that had been micropropagated for at least five years (Ulrich et al. 2008a and b). One of the predominant endophytic strains that was investigated in detail grew well under the special in vitro conditions and clearly enhanced the development of the roots. Poplar microcuttings inoculated with this *Paenibacillus* strain showed significantly more roots and higher root length compared with

control plants (Ulrich et al. 2008a). Bacteria of the genus *Paenibacillus* have been found as endophytes in various other trees such as pine and coffee (Bent and Chanway 2002; Sakiyama et al. 2001). Species of this genus are known to produce plant hormones such as auxin and cytokinin (Lebuhn et al. 1997; Timmusk and Wagner 1999), peptide antibiotics (Beatty and Jensen 2002), as well as different hydrolyzing enzymes (Park et al. 2007; Sakiyama et al. 2001), and thus belong to the “plant-growth-promoting bacteria”.

In this study, the influence of this endophytic *Paenibacillus* isolate on the metabolism of poplar shoot explants, free from culturable endophytic bacteria, was analyzed via gas chromatography coupled to time-of-flight mass spectrometry (GC-TOF-MS). The technique is able to analyze a high dynamic range of metabolite abundance and high-quality peak shapes and spectra, which allows the deconvolution of co-eluting compounds (Glinski and Weckwerth 2006; Weckwerth 2008). Although not covering the complete metabolome, GC-TOF-MS is, at present, a widely applied technique for diagnostic biomarker discovery and sample pattern recognition (Morgenthal et al. 2005; Weckwerth and Morgenthal 2005) as well as for the investigation of plant–microbe interactions in nodules (Barsch et al. 2006a and b; Desbrosses et al. 2005). Knowing that our *Paenibacillus* sp. isolate has a beneficial effect on root growth in poplar cultures and is an N-fixing bacterium (Ulrich et al. 2008a and b), we hypothesized a strong influence of this microbe on poplar central C/N metabolism. Metabolomics was used to comprehensively investigate the effect on plant carbon metabolites as being the major substrates and products involved in N assimilation processes.

RESULTS

Endophyte-free poplar in vitro cultures and inoculation with an endophyte isolate *Paenibacillus* P22.

The effect of endophytic *Paenibacillus* spp. on plant metabolism was tested using in vitro explants of the Chinese hybrid poplar clone 741 (♀ [*Paenibacillus alba* × (*P. davidiana* + *P. simonii*) × *P. tomentosa*]). By regeneration of shoot meristems from glasshouse-grown plants, explants were produced free from culturable bacteria. The first growing shoots from isolated meristems were obtained after 1 month. Shortly after reaching a length of approximately 1.5 cm, some of the shoots were homogenized, diluted with 0.85% NaCl, and plated on R2A to test for bacterial-free state.

For inoculation of explants, the *Paenibacillus* isolate 22 was used. This isolate was obtained from a micropropagated cul-

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*The e-Xtra logo stands for “electronic extra” and indicates that Figures 1, 2, and 3 appear in color and a supplementary table is published online.

ture of poplar clone 741 two years ago. The strain showed a strong similarity to *P. humicus* (99.5%) according to phylogenetic analysis based on 16S rRNA gene sequences. Endophytic bacteria belonging to this *Paenibacillus* group were found in different in vitro cultures of various poplar, larch, and spruce clones (Ulrich et al. 2008a and b).

Poplar microcuttings inoculated with the endophytic *Paenibacillus* isolate 22 showed significantly more roots per cutting and higher root length compared with the control plants after 3 weeks (Ulrich et al. 2008a). In addition, the sprouts of plants without bacteria appeared slightly vitreous.

Metabolite analysis of poplar plants with and without *Paenibacillus* inoculation after 3 weeks.

For metabolomic analysis, shoots of five control plants and inoculated plants were sampled in two different independent experiments. Metabolite analysis was performed with GC-TOF-MS according to protocols of Weckwerth and associates (Morgenthal et al. 2005, 2007; Weckwerth et al. 2004).

To reduce data dimensionality after metabolite profiling, we applied multivariate data analysis in the form of principal and independent component analysis (PCA/ICA) as an unsupervised analysis for rapid diagnostics of sample pattern. Based on the identification and quantification of approximately 70 compounds, the PCA/ICA separated the samples into two groups: poplar clones with and without endophytic bacteria (Fig. 1). The comparison between two independent replications of the experiment (A and B) resulted in a similar PCA/ICA pattern, indicating similar metabolite behavior. The changes of the metabolite levels between poplar plants with and without inoculation for both experiments were visualized by *t* test clustering (Fig. 2). Significant changes of 32 metabolites in experi-

ment A and 15 metabolites in experiment B were determined. In total, 11 metabolites showed equally significant changes in both experiments. Several intermediates such as organic acids (malate, succinate, fumarate, and citrate), amino acids (phenylalanine, 2-ketoglutarate, and oxo-proline), and sugar-phosphates (fructose-6-P) were significantly decreased in the inoculated plants in both experiments, whereas only asparagine, urea, and threitol were significantly increased. Inoculation with the endophytic strain resulted in the induction of urea up to sixfold and of asparagine up to eightfold. Although experiment A had more significant changed metabolites compared with experiment B, differences between the poplar clones with and without endophytic bacteria were reproducible in both experiments.

DISCUSSION

The combination of metabolite profiling and multivariate data mining is a powerful approach to classify samples and to reveal key metabolites responsible for specific processes. Here, we applied this technology to verify the hypothesis that poplar plants with and without endophytes have different metabolic signatures. To ensure that we indeed measured plant metabolites and not a mixture of bacterial and plant metabolites, bacterial population densities were determined, which ranged from 4×10^6 to 1.2×10^7 CFU g⁻¹. The corresponding biomass ratio was 1 g of plant material to 1×10^{-5} g of bacteria. Consequently, the contribution of the bacteria biomass is far below the detection limits of our GC-TOF-MS analysis and we have measured exclusively plant metabolites.

The hypothesis that the inoculation with *Paenibacillus* spp. significantly changed the metabolic composition of in vitro-

Fig. 1. Principal component and independent component analyses (PCA/ICA) of metabolites obtained from *Paenibacillus*-inoculated (orange or brown points) and noninoculated (dark- or light-green points) poplar plants in two independent experiments. Four replicates for each experiment were analyzed. PCA/ICA with normalized metabolite data ($x = [\text{value} - \text{average}] / \text{standard deviation}$), and the first three principal components include 93% of total variance. Each visualized point represents three technical measurements with 70 determined metabolites.

A similar effect is suggested for rhizobia in legumes by Prell and Poole (2006), constituting a model of metabolic exchange of carbon sources (malate, succinate, or fumarate) and amino acid cycling. The metabolic response of interaction between the endophyte P22 and poplar seems to work in a similar way.

In another context, this novel endophyte–plant interaction becomes interesting. Recently, *Azoarcus* spp. grass endophytes were shown to have plant-growth-promoting effects (Hurek et al. 2002). This mutualistic interaction is suggested to play an important role in natural grass ecosystems. This grass endophyte was recently sequenced, revealing a putative mechanism of plant–microbe interaction (Krause et al. 2006).

In conclusion, this study revealed an unambiguous metabolic signature of the mutualistic interaction of the endophytic *Paenibacillus* strain on metabolism of micropropagated poplar plants. These results present the first application of metabolomics to such a highly controlled in vitro growth system, thereby providing a means to investigate endophyte–plant interaction precisely. Further work will be performed to study this interaction in more detail as well as the consequences of the identified metabolic changes.

MATERIALS AND METHODS

Reagents.

Chemicals were purchased from Sigma (Taufkirchen, Germany), except D-sorbitol- $^{13}\text{C}_6$, DL-leucine-2,3,3- d_3 , and L-aspartic acid-2,3,3- d_6 , which were obtained from Isotech (Miamisburg, OH, U.S.A.).

Growth of endophyte *Paenibacillus* P22 in nitrogen-free medium.

The *Paenibacillus* isolate 22 was obtained from a micro-propagated culture of poplar clone 741 two years ago. The strain showed a strong similarity to *P. humicus* (99.5%) according to phylogenetic analysis based on 16S rRNA gene sequences and could be found in different in vitro cultures of other woody plants, too (Ulrich et al. 2008a). No other bacteria were found in the cultures after two years of propagation, also according to Leifert and associates (1989).

Growth of *Paenibacillus* P22 was tested on nitrogen-free medium according to Ma and associates (2007).

Growth of endophyte-free poplar in vitro cultures.

Endophyte-free poplar shoots were generated from in vitro explants of the Chinese hybrid poplar clone 741 (♀ [*P. alba* × (*P. davidiana* + *P. simonii*) × *P. tomentosa*], Prof. Han, Beijing, China). By regeneration of shoot meristems from glasshouse-grown plants, explants were produced, which were free from culturable bacteria. Meristems were removed from stem buds under a binocular microscope using a special microscalpel. The preparation was performed without prior disinfection of the branches to avoid contamination by hairs existing on the surface of this clone. The isolated meristems were placed on a modified Linsmaier and Skoog medium (L. Yang, *personal communication*) and incubated at 22°C under a 16-h photoperiod ($28 \mu\text{mol m}^{-2} \text{s}^{-1}$) to regenerate plants. The first growing shoots were obtained after approximately 1 month. Shortly after reaching a length of approximately 1.5 cm, samples of the shoots and roots were homogenized, diluted with 0.85% NaCl,

Fig. 3. Metabolic signature of the mutualistic interaction of the endophyte *Paenibacillus* sp. strain P22 and in vitro-grown poplar plants. Significant metabolite changes to central plant metabolism are shown as a response to endophyte infiltration of poplar tissue cultures. These metabolic changes were reproduced in two independent experiments.

and plated on trypton soya agar (TSA) medium to test for the bacteria-free state. Prior to the experiments, the plants were also checked.

***Paenibacillus* inoculation of endophyte-free poplar in vitro cultures.**

The inoculation was performed by exposing the basal 5-mm stem part of the microcuttings (20 mm in length) to an overnight TSA broth culture of isolate 22 (final concentration approximately 2×10^7 CFU ml⁻¹) for 2 s before planting them on the in vitro medium BEMB₂₀₀ (Ewald and Suss 1993). The control cuttings were given a quick dip in TSA medium.

Three weeks after inoculation, the shoots of the plants were harvested for metabolic analysis. The colonization of *Paenibacillus* isolate 22 in the infected plants was determined by grinding roots and shoots of five representative plants in 0.85% NaCl and plating on TSA. The calculated population densities ranged from 4×10^6 to 1.2×10^7 CFU g⁻¹. The corresponding biomass ratio is 1 g of plant material to 1×10^{-5} g of bacteria.

Extraction and derivatization.

The plant material (8 to 10 mg of the frozen samples) was homogenized and extracted using methanol/chloroform/water (2.5:1:0.5, vol/vol/vol) (Weckwerth et al. 2004). For data normalization and quality control, 10 µl of internal standard solution (leucine-2,3,3-d₃, aspartic acid-2,3,3-d₃, and D-sorbitol-¹³C₆) was added. After fractionation, the upper polar phases were used for GC-MS analysis and dried completely in a speed-vac concentrator. The residue was dissolved and derivatized for 90 min at 30°C (20 µl of methoxyamine hydrochloride at 40 mg/ml in dry pyridine) followed by a 30-min treatment at 37°C (with 90 µl of *N*-methyl-*N*-trifluoroacetamide). The retention time index marker (3 µl) (n-alkanes C10 to C36, each 200 µg/ml in pyridine) were added to every sample to determinate the retention time index (Morgenthal et al. 2007).

GC-TOF-MS.

For GC-TOF-MS analysis, a typical setup for plant metabolite profiling via GC coupled to a LECO Pegasus IV TOF (Leco Corp Inc., St. Joseph, MI, U.S.A.) mass analyzer was used according to Weckwerth and associates (Morgenthal et al. 2005, 2007; Weckwerth et al. 2004).

The GC-TOF-MS analysis was performed on an HP 6890 gas chromatograph with deactivated standard split/splitless liners (Agilent, Böblingen, Germany).

Sample volumes of 1 µl were injected in splitless mode at 230°C injector temperature. GC was operated on a VF-5ms capillary column, 30 m long, with a 0.25-mm inner diameter, 25-µm film thickness, and 10-m EZ-guard precolumn (VAR-1AN, Palo Alto, CA, U.S.A.) at constant helium flow of 1 ml/min. The temperature program started with 1 min isocratic at 70°C followed by temperature ramping at 9°C/min to a final temperature of 350°C, which was held for 5 min. Scan rates were set to 20 s⁻¹ and mass ranges set to 71:500 mass-to-charge ratio (m/z).

Data analysis and statistics.

The measured data were analyzed with the ChromaTof software from LECO performing automated deconvolution of co-eluting compounds from a total ion chromatogram. The data processing includes mass-spectral correction for co-eluting metabolites and retention index calculation based on n-alkanes. Seventy polar metabolites, mainly from the primary metabolism, were annotated and defined in a reference chromatogram. Afterward, all chromatograms were matched against the reference with a minimum match factor of 800. Com-

pounds were annotated by retention index and mass spectra comparison to the GMD library (Kopka et al. 2005). Selected fragment ions specific for each individual metabolite were used for peak area quantification. Each compound was normalized by the peak area from the internal standard and by the fresh weight of each sample. These relative response ratios can be directly compared between different samples without knowledge of absolute compound concentrations. Finally, the processed data were exported and aligned by the software tool MetMax (GC-GC-MS Data Matrix Creator) (Kempa et al. 2009). For statistical analysis, the TIGR MeV software (version 4.1) was used to perform PCA and *t*-test clustering. Further, PCA/ICA was performed according to Morgenthal and associates (2005, 2007). Metabolite contributions to the components were quantified through the component scores and loadings. For each PCA/ICA, the data were log-transformed. The *t* test clustering was performed with Welch approximation between subjects (poplar + and – endophytes). The *t* values were calculated for each metabolite and *P* values based on *t* distribution. Significant differences in the metabolite level between the two groups were determined by comparing the *P* values (critical $\alpha = 0.05$). The clustering was computed with K-mean and Euclidian distance, which groups metabolites by degree of similarity (or dissimilarity). Data normalization was calculated with the formula $x = (\text{value} - \text{average})/\text{standard deviation}$.

ACKNOWLEDGMENTS

We thank H. Enkisch for excellent technical assistance, L. Yang from the Agricultural University Hebei, Baoding for providing the poplar clone 741, and S. Wienkoop for fruitful discussion and advice.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Institute for Genomic Research MultiExperiment Viewer software: www.tm4.org