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Characterization of the *Medicago truncatula* cell wall proteome in cell suspension culture upon elicitation and suppression of plant defense

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ABSTRACT

In addition to establishing methods for proteome analysis of cell wall proteins (CWPs) for the model plant *Medicago truncatula*, this work highlights the presence of several protein classes in cell culture. Using a combination of two-dimensional gel electrophoresis (2D-PAGE) and/or liquid chromatography–tandem mass spectrometry (LC–MS/MS), we established the proteome reference map of *M. truncatula* cell wall proteins. CWPs extracted from purified cell wall fragments resulted in the identification of 46 (2D-PAGE) and 65 (LC–MS/MS) proteins, respectively, with a total of 111 proteins. The identified proteins are involved in various processes, including cell wall modifications, signaling, defense mechanisms, membrane transport, protein synthesis and processing.

Further, we conducted comparative proteome analysis to identify changes in protein composition during interaction of *M. truncatula* cell suspension culture with a pathogen-derived yeast elicitor (YE) and suppressor using *Sinorhizobium meliloti* LPS. 2D-PAGE analysis for the CWPs after YE and LPS treatment resembled the proteome map of YE alone, with a few up-regulated proteins involved in defense, and in the case of the LPS-treated cell wall proteome, there was no significant difference observed. Using this approach, proteins involved in defense, such as L-ascorbate peroxidase, specifically targeted proteins to the cell wall during defense, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and proteins that play an important role during growth and development were identified. Also, some defense-related proteins were absent in the same gel after YE treatment, suggesting that oxidant protection is regulated by these proteins.

Introduction

Rigid cell walls are characteristic features of plants. They are essential, and perhaps the most prominent, factors responsible for the architecture of the entire plant. During the last decade, it has become obvious that plant cell walls carry out many diverse functions in addition to their prominent role as an extracellular skeleton for the whole individual. The plant cell wall is a dynamic structure whose content changes in response to internal and external factors (Hueckelhoven, 2007). Three major polymeric components of the cell wall, the polysaccharides, the phenolics and the proteins have been shown to change both quantitatively and qualitatively in response to a variety of signals from the plant and the environment (Carpita and Gilbeaut, 1993; Yong et al., 2005). The cell wall changes in composition during expansion growth, differentiation and in response to environmental stress and pathogenic attack (Carpita and McCann, 2000; Jamet et al., 2006; Lee et al., 2004; Cho et al., 2009). Biotic and abiotic stresses induce characteristic changes in cell walls that allow the plant to adapt to different situations as needed. Plants exhibit natural resistance to disease, which involves phytoalexin synthesis, wall toughening, accumulation of the cell wall hydroxyproline-rich glycoproteins (HRGPs) and increased activity of lytic enzymes such as chitinases and other antimicrobial proteins found in plant cell walls (Bowles, 2007; Saikia et al., 2005). Such responses can be induced not only upon infection through living microbes, but also by microbial and endogenous plant elicitors (Radman et al., 2003; Templeton and Lamb, 1988).

Initial elicitation studies in plant cell systems, and the name 'elicitor' were introduced by Keen (1975) and Albersheim et al. (1977). They investigated the interaction of microbial oligosaccharides with plants. Elicitors are the compounds released by microorganisms or liberated from the cell walls by microbial enzymes that are responsible for the activation of defense responses. Elicitors induce several signaling pathways, beginning

Abbreviations: 2D-PAGE, two-dimensional gel electrophoresis (2D-PAGE); CWPs, cell wall proteins; LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; MALDI-TOF MS, matrix assisted laser desorption ionization-time of flight mass spectrometry; *Mt*, *Medicago truncatula*; PMF, peptide mass fingerprinting; YE, yeast elicitor.

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with ion fluxes, the oxidative burst reaction and synthesis of signaling molecules such as jasmonic and salicylic acids as secondary messengers (Katz et al., 2002). Defense gene activation, which in some cases occurs within a few minutes upon elicitor treatment, is part of a massive change in the pattern of mRNA, and consequently to protein synthesis underlying the induction of defense responses (Cramer et al., 1985). Among the early genes transcribed upon elicitations are genes coding for transcription factors (Harrison, 1998; Naoumkina et al., 2008). Elicitors have also been used as tools to understand the complex pathways and interaction of plant secondary metabolites.

In addition to pathogen attack, many plants are exposed to symbiotic microbes. Most, and perhaps all, plants establish some kind of symbiotic interaction with other organisms. The most well-studied symbiotic interactions are mycorrhizal symbiosis (Harrison, 1998) and nitrogen-fixing *Rhizobium*-legume symbiosis (Hirsch, 1992). In the case of *Rhizobium*-legume symbiosis, the rhizobia enter the host plant via the root hairs through the formation of tubular structures called infection threads (Mergaert et al., 2006). Rhizobia induce a novel plant organ, the so-called root nodule. Within these structures, plant cells are infected by the invading microbes. The bacteria differentiate within a short period of time into nitrogen fixing bacteroids (Hirsch, 1992). For various *Rhizobium* species, it was found that exopolysaccharides (EPS) and lipopolysaccharides (LPS) play an important role in the infection of the host plant (Albus et al., 2001). The rhizobial cell surface components involved in the plant-bacterial interaction were the EPS and LPS bound to the outer membrane of Gram-negative bacteria (Zevenhuizen et al., 1980; Carlson, 1984). *Sinorhizobium meliloti* LPS has been reported previously as a suppressor of the elicitor-induced oxidative burst reaction in *Medicago sativa* cell cultures (Albus et al., 2001; Tellstroem et al., 2007). In addition, *S. meliloti* LPS acts as a specific signaling molecule, promoting the symbiotic interaction and suppressing a pathogenic response in the host plant alfalfa (Albus et al., 2001).

Medicago truncatula, a close relative of alfalfa, has been used as a model legume because of its small diploid genome, self-fertility and relatively easy transformation (Cook, 1999). Additionally, the large-scale sequencing of *M. truncatula* ESTs allows us a chance to study the variation in the plant cell wall proteome in response to both symbiotic and pathogenic interaction.

As plant cells cultured in suspension have cell walls comparable to primary cell walls found in the meristematic cells of the plant, they can be used to study some of the signal-dependent changes in their composition. Similarly, changes in the wall components due to the action of stress-inducing compounds such as elicitors (Bolwell et al., 1985; Robertson et al., 1997) or suppressors (Albus et al., 2001) can be analyzed. *M. truncatula* suspension cell cultures grown in liquid MS medium were used to identify differences in cell wall protein profiles during different treatments. The present work focuses on the effect of YE and LPS on *M. truncatula* cell wall proteins. We used yeast invertase as elicitor and LPS of *S. meliloti* as a suppresser of host cell defense reactions. The cell wall proteins (CWPs) extracted after these treatments were analyzed by 2D-PAGE and LC–MS/MS.

Materials and methods

Cell cultures

The *Medicago truncatula* suspension cell culture was grown in liquid MS medium (Murashige and Skoog, 1962) by shaking on an orbital platform at 120 rpm. 90 mL of cells were routinely transferred to 110 mL fresh MS medium in 1 L Erlenmeyer flasks every week. The cell cultures were maintained at 20–24 °C in the dark. Suspension cells of 5-day-old cultures were used for the experiments.

Hot phenol extraction and purification of LPS

Sinorhizobium meliloti wild-type strain 2011 cells were grown on TY agar plates for 3 days and washed from the plates with 0.9% NaCl (w:v). After centrifugation at 5000 × *g* for 20 min, cell pellets of approximately 60 g wet weight were resuspended in H_2O and LPS was extracted using the hot phenol–water method (Westphal and Jann, 1965). The water phase was dialyzed extensively against water. Proteins and nucleic acids were removed by treatment of the dialysate with 100 μg/mL DNase I (Boehringer Mannheim, Germany), 15 μg/mL RNase (Boehringer Mannheim) and 150 μg/mL proteinase K, redialyzed and lyophilized. The LPS was resuspended and purified by ultracentrifugation at 100,000 × *g*. Subsequently, the LPS was further purified by gel-permeation chromatography using a Sephadex G-50 matrix in a pyridine acetate solvent (0.4% pyridine, 1% acetic acid).

Determination of the oxidative burst reaction in M. truncatula cell suspension cultures

The detection of the oxidative burst from 5-day-old *M. truncatula* cell cultures was performed using the H_2O_2-dependent chemiluminescence reaction, as described previously (Scheidle et al., 2005).

Elicitor and suppressor treatment

For the suppression of YE-induced oxidative burst activity, *S. meliloti* LPS was used. The *M. truncatula* cell cultures were treated with water, 25 μg mL^{-1} YE (Sigma), 20 μg mL^{-1} *S. meliloti* LPS, or a combination of 25 μg mL^{-1} of YE and 20 μg mL^{-1} *S. meliloti* LPS, were incubated for 90 min and 12 h with constant shaking.

Cell wall protein extraction and sample cleanup

Protein extraction was made from *M. truncatula* suspension cell culture according to Watson et al. (2004). The $CaCl_2$ extracts were pooled and concentrated. Furthermore, the samples were resuspended in 8 M urea and 4% CHAPS. Protein concentrations were determined using the Bradford method with BSA as the standard.

Two-dimensional gel electrophoresis

For isoelectric focusing (IEF) as a first dimension, 750 μg of proteins in 400 μL rehydration buffer containing 5 μL of 28% (w/v) DTT and 1% final concentration of IPG buffer pH 3–10 (Amersham Biosciences) were loaded directly onto pH 3–10 IEF 24 cm gel strips (Immobiline DryStrip, Amersham Biosciences). IEF was performed for 20–24 h to 75,000 Vh using the IPGphor™ system (Amersham Biosciences). After focusing, the proteins were reduced for 15 min in 5 mL of equilibration buffer I (50 mM Tris/HCl, pH 8.8, 6 M urea, 30%, v/v glycerol, 2% SDS, 0.01%, w/v bromophenol blue) containing 50 mg of DTT and alkylated for another 15 min in 5 mL of the same buffer with 225 mg of iodoacetamide. The second dimension was performed using 12.5% polyacrylamide gels (200 mm × 200 mm × 1 mm) using an Ettan Dalt 2D-gel system (Amersham Biosciences). The gels were fixed in 10% (v/v) acetic acid, 30% ethanol (fixing solution) for 30 min, stained for 90 min in a Coomassie Brilliant Blue (CBB) staining solution (5%, v/v methanol, 42.5%, v/v ethanol, 10%, v/v acetic acid, 30%, v/v ethanol, 0.2%, w/v CBB G250, 0.05%, w/v CBB R250) and de-stained in fixing solution twice for 60 min. The gels were further de-stained in 7% (v/v) acetic acid until the backgrounds were clear.