Suppression of defense-associated gene expression

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The lipopolysaccharide of *Sinorhizobium meliloti* suppresses defense-associated gene expression in cell cultures of the host plant *Medicago truncatula*

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Abstract

In the establishment of the symbiosis between *Medicago truncatula* and the nitrogen-fixing bacterium *Sinorhizobium meliloti* the lipopolysaccharide (LPS) of the microsymbiont plays an important role as a signal molecule. It has been shown in cell cultures that the LPS is able to suppress an elicitor induced oxidative burst. To investigate the effect of *S. meliloti* LPS on defense-associated gene expression a microarray experiment was performed. For the evaluation of the *M. truncatula* microarray datasets the software tool MapMan that was initially developed for the visualization of *Arabidopsis thaliana* datasets was adapted, by assigning Medicago genes to the ontology originally created for Arabidopsis. This allowed functional visualization of gene expression of *M. truncatula* suspension cultured cells treated with invertase as an elicitor. A gene expression pattern characteristic for a defense response was observed. Concomitant treatment of *M. truncatula* suspension cultured cells with invertase and *S. meliloti* LPS leads to a lower level of induction of defense-associated genes, compared to induction rates in cells treated with invertase alone. This suppression of defense-associated transcriptional rearrangement affects genes induced as well as repressed by elicitation and acts on transcripts connected to virtually all kinds of cellular processes. This indicates that LPS of the symbiont not only suppresses fast defense responses as the oxidative burst but also exerts long-term influences, including transcriptional adjustment to pathogen attack. These data indicate a role for LPS during the infection of the plant by its symbiotic partner.
Plants and animals have the capacity to recognize and attack pathogenic microorganisms such as fungi, viruses or bacteria. But in contrast to animals plants lack a system of circulating fluids like blood and lymph as well as specialized immune cells. Therefore, each plant cell has to possess the ability to recognize different pathogenic molecules and to respond with a broad range of defense reactions. In recent years, it has become more and more evident that there are two general systems of plant immunity (Gómez-Gómez and Boller, 2002; Nürnberger et al., 2004). The cultivar specific resistance is realised on a gene for gene basis. In this context, certain plant cultivars carrying a specific resistance gene are able to mount defense reactions against pathogenic microorganisms that carry a corresponding avirulence gene. These defense reactions usually include the induction of a hypersensitive response (HR) in order to inhibit the spread of a pathogen as fast as possible (Dixon and Harrison, 1994). The cultivar specific resistance is discussed to resemble the animals’ adaptive immune system although it does not involve specialized immune cells or recombinatory events on the genetic level. The second type of plant immunity is the ability to distinguish between self and non-self. This strategy is known from animals as innate immunity and meanwhile this term is also applied to the plant system (Gómez-Gómez and Boller, 2002). The recognition of foreign organisms in the plant’s surrounding often does not lead to such vigorous responses like the activation of a HR, but rather induces general preparations to a possible invasion by microorganisms. These defense mechanisms include the induction of an oxidative burst, the expression of defense related genes, the production of antimicrobial compounds like phytoalexins and reinforcement of the plant cell wall (Benhamou, 1996; Ebel and Mithöfer, 1998; Heath, 2000). Although these responses are also activated during the cultivar specific resistance the hypersensitive cell death is absent during basal defense. The plant’s innate immunity is based on the recognition of microbial signal molecules termed general elicitors or pathogen-associated molecular patterns (PAMPs). Unlike the products of avirulence genes in the cultivar-specific resistance, PAMPs are expressed by a wide range of microorganisms and are usually indispensable for their biological fitness. Among the PAMPs are long known and well characterised elicitors like (1,3-1,6)- hepta-β-glucoside isolated from cell walls of Phytophthora sojae (Sharp et al., 1984 a,b), chitin of higher fungis’ cell walls (Bartnicki-Garcia, 1968, Ren and West, 1992), yeast invertase (Basse et al., 1992), flagellin as the first general elicitor isolated from Gram-negative bacteria (Felix et al., 1999) or lipopolysaccharides (LPS) (Meyer, 2001; Erbs and Newman, 2003). The potential of LPS to induce defense reactions is well characterized for the
animal system but also described for plants. Pre-treatment with LPS from different bacteria has been shown to prevent HR and to accelerate the production of salicylic acid in host as well as non-host plants (Graham et al., 1977; Newman et al., 1997; Newman et al., 2002). Furthermore, Xanthomonas campestris pv. campestris LPS is able to induce a defense related gene expression, the oxidative burst as well as calcium signalling in tobacco suspension cultures (Meyer et al., 2001; Braun et al., 2005; Silipo et al., 2005). Using LPS of Burkholderia cepacia the induction of several early signalling and defense pathways in tobacco has been confirmed (Gerber et al., 2004). Recent studies have identified several tobacco proteins altered in their phosphorylation status in response to elicitation with B. cepacia LPS, giving valuable insights into possible signalling processes that follow the perception of the PAMP (Gerber et al., 2006).

Lipopolysaccharides are also important signal molecules in symbiotic signalling. In the Rhizobium-legume symbiosis evidence for the involvement of LPS in the establishment of symbiosis has been gained from mutants defective in LPS synthesis. In different kinds of Rhizobia-legume associations alterations in the LPS structure lead to delayed nodulation, the abortion of infection threads, formation of non-fixing nodules and induction of plant defense reactions (Carlson et al., 1987; Lagares et al., 1992; Perotto et al., 1994; Niehaus et al., 1997; Campbell et al., 2002). In 2001 Albus et al. showed, that LPS of the microsymbiont Sinorhizobium meliloti is able to suppress an elicitor induced oxidative burst in suspension cultures of the host plant Medicago sativa. Since this LPS does induce an oxidative burst in cell cultures of the non-host plant Nicotiana tabacum the suppression effect is not a general feature of the rhizobial lipopolysaccharide but is speculated to be a very specific feature in the establishment of a symbiotic relationship. Recent results identified the LipidA substructure of the S. meliloti LPS to be the suppressor-active portion (Scheidle et al., 2005). However, the extent to which rhizobial LPS is able to suppress the host’s defense mechanisms has remained poorly characterised. To gain more information on the capacity of S. meliloti LPS to interfere with the plants defense machinery we performed microarray experiments in order to monitor the global expression of defense related genes. Defence reactions were induced by yeast invertase (Basse et al. 1992). By treatment of suspension cultured Medicago truncatula cells with either S. meliloti LPS and yeast invertase or yeast invertase alone, the effect of S. meliloti LPS on the level of gene expression could be addressed. The comparison of defense related gene expression in the two kinds of treatment showed, that the induction of many genes strongly correlated to plant defense (e.g. phenylalanine ammonia lyase and isoflavone reductase) was significantly suppressed after LPS pre-treatment. Using the MapMan software
(Thimm et al., 2004; Usadel et al., 2005) it was furthermore possible to make a functional correlation between the suppression effect calculated for all genes displayed on the microarray and their specific function inside the cell.

Results and Discussion

Invertase induces defense associated gene expression in *Medicago truncatula* cell suspension cultures

In this study, *M. truncatula* suspension cultured cells derived from roots were used as a test system for the perception of microbial signal molecules. In order to verify the responsiveness of the cell culture invertase was used as a well established elicitor of plant defense reactions. In all 4 biological replicates used, the addition of invertase to a final concentration of 50 µg mL⁻¹ lead to the production of hydrogen peroxide with an average maximal concentration (± SE) of 3.54 µM H₂O₂ (± 0.15) 15 min after elicitation (Fig.1). The oxidative burst assay was used as a test system for the uniform perception of the elicitor in the different experiments. Only responsive cell cultures were used for subsequent expression profiling.

For transcriptome profiling studies, *M. truncatula* cell suspensions were treated for four hours with either invertase, *S. meliloti* LPS, invertase + *S. meliloti* LPS or water as a control. Cells were harvested, frozen and processed for transcriptome profiling. Based on the analysis of all four biological replicates in comparison to water treated suspension cells, LPS treatment alone did hardly show any effect on gene expression in *M. truncatula* cell cultures. A complete list of genes meeting the prerequisites $p \leq 0.05$, $A \geq 5.5$ and $n \geq 6$ ($p$, $A$ and $n$ describe the statistical relevance, signal intensity and the number of agreeing replicates, respectively) is included in Supplemental Table I. No gene in this list was identified as induced 2-fold or more and only 2 genes (TC88927 and TC78069) were repressed more than 2-fold. These results indicate that *M. truncatula* cell cultures do not respond strongly to the LPS of the symbiotic partner. In contrast, in suspension cells treated with invertase 336 genes were identified to be at least 2-fold induced with a statistical significance of $p \leq 0.05$, whereas 43 genes were identified to be repressed at least 2-fold. Table I lists the 20 genes which are most strongly induced or repressed, respectively and the complete list of genes meeting the prerequisites $p \leq 0.05$, $A \geq 5.5$ and $n \geq 6$ is included in Supplemental Table I.

14 of the 20 most strongly induced genes are involved in plant secondary metabolism, e.g., isoflavone reductases (IFR), chalcone synthases (CHS), chalcone reductase (CHR) and phenylalanine ammonia lyase (PAL). The induction of the secondary metabolism is a plant
defense response well described for alfalfa, a very close relative of *M. truncatula* as well as for other plant systems (Kuhn et al., 1984; Dalkin et al., 1990; Ni et al., 1996; Suzuki et al., 2005; for a recent review see Dixon et al., 2002). PAL is the branch point enzyme transforming L-phenylalanine to *trans*-cinnamic acid a precursor molecule for the formation of lignin as well as the isoflavonoid-derived phytoalexin medicarpin (Dalkin et al., 1990). The biosynthesis of the latter compound also involves the activity of CHS, CHR and IFR. Another gene strongly induced after elicitation with invertase was a chitinase, involved in the defense against fungal pathogens. Furthermore, transcripts encoding two germin-like proteins were highly abundant in the elicited cell cultures. Germin-like proteins are ubiquitous plant proteins encoded by diverse multigene families. Although the function of most Germin-like proteins could not be elucidated so far, nectarin I a germin-like protein present in the nectar of tobacco plants was found to exhibit superoxide dismutase activity (Carter and Thornburg, 2000). Additionally, a gene encoding a cyanogenic beta-glucosidase was strongly induced after elicitation. Cyanogenesis has been described in the context of protection against herbivores but due to the high toxicity of HCN other roles in plant defense were postulated (Poulton, 1990).

6 of the 20 most repressed genes encode proteins involved in cell wall degradation. Since the reinforcement of the plant cell wall is one of the major responses upon pathogen attack it was not unexpected to find the expression of degradative enzymes repressed.

Manual classification of the transcription profiling data according to published literature revealed that among the 336 genes induced 2-fold or more after elicitation with invertase more than 170 have been described to be involved in plant defense or to be induced under biotic stress conditions. Grouping of all genes induced at least 2-fold into functional categories enables the illustration of different plant responses upon elicitation (Fig.2). These include the expression of several signalling components, the activation of different hormone pathways, the expression of proteins involved in protection from oxidative stress as well as the induction of transcriptional regulators. Besides the two largest categories that are composed of genes with unknown function and those which did not fit into any of the given functional categories (“others”) the two categories “secondary metabolism” and “biotic stress” comprise 14 and 16% of all genes induced at least 2-fold, respectively. These findings are in general agreement with previous studies (Suzuki et al., 2005) and confirm the responsiveness of the utilised *M. truncatula* suspension cell line on the transcriptome level.
The visualization tool MapMan allows fast and easy evaluation of transcriptome profiling datasets on the functional level

Transcriptome profiling studies can give valuable information on global cellular responses, however, the evaluation and interpretation of the datasets is time consuming and requires detailed knowledge of the existing literature. Bioinformatic tools can help to organize the datasets according to preexisting biological knowledge and therefore enable a comprehensive interpretation on the functional level. MapMan is a visualization tool developed to display plant genomic datasets onto pictorial diagrams (Thimm et al., 2004). The software organizes genes into blocks of cognate functions to allow even tentatively assigned genes and individual members of gene families to be displayed. This structure allows the detection of general trends in the systems response, and in cases where the ontology is further developed, preciser resolution at the single pathway level. Recently, MapMan has been extended to enable the statistical evaluation of differences in the responses of sets of genes assigned to different biological functions allowing more validated interpretation of global cellular responses (Usadel et al., 2005). Since MapMan was initially designed to process datasets obtained from Arabidopsis ATH1 microarrays, the adaptation of this tool for the employment with other microarray formats and plant species was an important step. In the beginning of 2006 Urbanczyk-Wochniak et al. published the extension of MapMan for displaying Solanaceous datasets. The basis for this adaptation is the classification of the genes represented on the microarray according to the ontology used in MapMan.

In order to use MapMan as a tool to visualize transcriptomic datasets obtained from M. truncatula Mt16kOLI1 and Mt16kOLI1Plus microarrays, we assigned the 16470 TCs represented on the Mt16kOLI1Plus array to MapMan BINS and subBINS based on a combination of automated BLAST searches against the Arabidopsis TAIR 6.0 database and protein domain information. TCs lacking a good hit in A. thaliana were assigned manually according to their TIGR annotation. It has to be mentioned that the ongoing sequencing efforts and annotation updates providing new information on the M. truncatula genome lead to an urgent need to constantly update the classification of the set of TCs. Furthermore, M. truncatula as a legume plant poses a challenge in the need to assign genes with a function in nodulation, a class of genes that is absent in Arabidopsis. To account for this legume specific class of genes, a new subbin named “nodulins” (subbin 26.31) was defined. Even though this BIN was not displayed in the visualization of the datasets presented here, it is a crucial prerequisite for the evaluation of data obtained from symbiotically active Medicago plants.
Since MapMan is designed to allow for the creation of user defined diagrams and visualization maps, this tool is well suited for the employment in a symbiotic context.

To demonstrate the value of the visualization of transcriptome data using MapMan the dataset obtained from invertase treated suspension cell cultures described above was used for a proof of concept study. In Figure 3 two different images are shown one representing a general overview of the cells basic metabolic pathways (a) and the other displaying different cellular responses (b). As discussed above, the induction of the secondary metabolism is one of the most noticeable responses in elicited *M. truncatula* cell cultures. The visualization of the cells general metabolism confirms this cellular response (Fig 3a). Furthermore, the organisation into hierarchical modules of biological relevance facilitates a more detailed resolution of the global responses. For example, the three modules representing the secondary metabolism pathways “flavonoids”, “phenylpropanoids and phenolics” as well as “N-misc” are clearly induced in this MapMan visualization. The strong induction of the pathways involved in lignification and phytoalexin synthesis was detected by standard evaluation methods as described above, whereas, the induction of the alkaloid metabolism (“N-misc”) had remained undetected. According to the *M. truncatula* TIGR Gene index 7.0 most Medicago genes contained in this BIN cannot be assigned unambiguously to a specific cellular function rendering the detection of an induction in alkaloid biosynthesis via standard evaluation methods impossible. Nevertheless, the automatic assignment of Medicago genes to the corresponding BIN as described in materials and methods leads to the identification of otherwise undetected response patterns such as the induction of the alkaloid pathway highlighting the applicability of the gene classification used to employ MapMan for the evaluation of Medicago datasets. The stimulation of alkaloid formation upon elicitation with fungal signal molecules has been shown before for different plant species (Blechert et al., 1995). Although, the production of alkaloids in Arabidopsis is poorly understood, the induction of the berberine biosynthetic pathway upon wounding was described (Cheong et al., 2002). Therefore, an induction of the alkaloid metabolism upon elicitation can also be expected in *M. truncatula*. Another conspicuous response of the invertase treated cell cultures is the transcriptomic rearrangement of cell wall metabolism. In this context, the mixture of induced and repressed genes can be easily interpreted by a closer examination of this module. Genes encoding for pectin methylesterases (PMEs) were induced upon elicitation with invertase. The transcriptional activation of PMEs upon herbivore attack has been shown before (von Dahl et al., 2006). In this context, demethylation of the cell wall pectin has been
proposed to enable cross linking via calcium ions resulting in the reinforcement of the cell wall. Other enzymes transcriptionally upregulated upon invertase treatment are cellulose synthases and polygalacturonase inhibitor proteins, whereas proteins involved in cell wall degradation, i.e., cellulases, polygalacturonases etc. are repressed. Cell wall reinforcement is known to be a crucial response upon pathogen attack, but a transcriptional initiation of this process besides the activation of lignifying enzymes or other secondary metabolic pathways has been rarely reported (Schenk et al., 2000). Another response that has been proposed to be induced following pathogen attack is the degradation of fatty acids by β-oxidation. Although the cluster of lipid metabolism did not show any marked changes closer examination of the transcriptional changes of this cluster reveal a minor induction of the β-oxidation pathway, as well as of the glyoxylate cycle. This is in accordance with a previous study that monitored transcriptional adaptations upon attempted infection of A. thaliana with Pseudomonas syringae pv. tomato (Scheideler et al., 2002). Although it is tempting to speculate that the mobilisation of fatty acids might be utilised for the generation of energy it should be emphasized, that on the other hand our data indicate a repression of starch degradation. This could hint to a role of β-oxidation in processes different from energy generation like the provision of precursor molecules for biosynthetic pathways. Furthermore, our data show an induction of the oxidative pentose phosphate pathway that has been shown to be essential in supplying NADPH for the activity of the NADPH oxidase during the oxidative burst (Pugin et al., 1997). Another response towards elicitation is the induction of the cluster representing the synthesis of aspartate family amino acids. This pattern is mainly caused by the induction of several S-adenosylmethionine synthetase enzymes involved in the production of the phytohormone ethylene that has been identified as one of the key players in orchestrating plant disease resistance (Feys and Parker, 2000). Finally, a trend in the induction of transcripts involved in the glutathione and ascorbate metabolism can be detected. Glutathione and ascorbic acid are indispensable for the protection of plant cells from oxidative stress. Even though the elicitor induced oxidative burst is an important element in plant defense and defense-associated signalling, the scavenging of reactive oxygen intermediates (ROI) is a crucial process to prevent oxidative damage of cellular compounds or the induction of programmed cell death (Mittler, 2002; Apel and Hirth, 2004). Figure 3b visualizes transcriptomic datasets with respect to different cellular responses. In this representation, the most striking response observed was for a class of genes assigned as involved in biotic stress. In fact, only five of these genes appear to be suppressed upon invertase mediated elicitation of cell cultures. Three of these are annotated as protease inhibitor proteins and could possibly be
involved in mechanisms different from the inhibition of proteases of pathogenic origin. Other patterns that can be observed are the induction of several genes involved in the response to different kinds of stress apart from drought and salt stress. Crosstalk between the responses towards different kinds of stresses is a frequent observation although a stronger induction of wound induced responses could have been expected (Desikan et al., 2001; Cheong et al., 2002). The class of miscellaneous genes involved in the response to abiotic stress contains four highly induced genes. All four are annotated as encoding germin-like proteins which are well known to be elicitor inducible proteins. The above discussed induction of genes involved in the protection from oxidative stress is visualised in more detail in figure 3 b demonstrating, that apart from the ascorbate and glutathione metabolism little transcriptional reprogramming can be observed upon elicitation. An induction of antioxidative proteins like dismutases and catalases is missing despite the potential need for scavenging ROI produced during the oxidative burst. This could hint towards a role of the ascorbate and glutathione cycle intermediates in addition to detoxification of ROI. It has been proposed, that glutathione itself is involved in signalling events leading to the activation of plant defense responses (Dron et al., 1988; Gomez et al., 2004). On the other hand a function as a superoxide dismutase has been described for a germin-like protein from tobacco nectar nectarin I. Although such a function remains purely speculative the above described induction of four germin-like proteins could be due to their function in the scavenging of ROI. Finally, some transcriptional rearrangements can be observed in the cluster of development associated genes showing predominantly a repression of this class. The transcriptomic adaptation of genes involved in cell maintenance and development has been described before (Schenk et al. 2000). However, no agreements on the single gene level could be made by comparing our results to the list of regulated genes described earlier.

In summary, the clear patterns of plant response upon elicitation visualized using MapMan are in accordance to existing literature and validate the usefulness of this software tool for the evaluation of transcriptome datasets. Nevertheless, it has to be emphasized, that the interpretation of the results lead to the identification of genes, that did not follow the general trend observed in the class they were assigned to. This can be explained either by opposing regulation of transcripts marking isoenzymes with different cellular functions or by the assignment of such genes have to a wrong BIN.
The lipopolysaccharide of the microsymbiont *Sinorhizobium meliloti* suppresses elicitor induced transcriptional reprogramming

In order to investigate the effect of *S. meliloti* lipopolysaccharide on defense associated gene expression in *M. truncatula*, we treated suspension cell cultures simultaneously with invertase and *S. meliloti* LPS with final concentrations of 50 µg mL\(^{-1}\), each. This concentration suppresses the invertase induced oxidative burst in *M. truncatula* suspension cultured cells to nearly 90% (Fig.1). In contrast, concomitant treatment of cell cultures with invertase and LPS of the phytopathogen *Xanthomonas campestris* pv. *campestris* did result in an oxidative burst with 91% maximal hydrogen peroxide production as compared to elicitation with invertase alone (Supplemental Figure I). These results indicate that the suppression effect is a specific feature of the *M. truncatula - S. meliloti* (legume - rhizobia) interaction which is further substantiated by the elicitation of an oxidative burst by *S. meliloti* LPS in tobacco cells (Albus et al., 2001). For transcriptional profiling analyses cells were incubated with invertase and *S. meliloti* LPS for 4 h, separated from the medium, frozen and RNA was extracted and processed for microarray hybridisations. Transcriptomic reprogramming induced by this treatment combining pathogenic and symbiotic stimulus was compared to the transcriptome profiles obtained by elicitation with invertase alone. Comparison of the two datasets was made possible by using the same RNA of water-treated cells as a reference. A list of all genes in the dataset obtained from the combined treatment meeting the prerequisites \(P \leq 0.05\), \(A \geq 5.5\) and \(N \geq 6\) is included in Supplemental Table I. 14 of the 20 most strongly induced genes are also among the 20 genes with highest induction rates after elicitation with invertase listed in Table I. Comparison of gene induction rates given in Table I shows that for all 20 genes most strongly induced upon invertase treatment there is also a strong induction following the combined treatment with invertase and *S. meliloti* LPS. Nevertheless, in each case the induction rate is markedly reduced giving a first hint toward a moderating activity of the rhizobial LPS. The same effect can be observed for 19 of the 20 genes most strongly repressed upon elicitation with invertase. To investigate general patterns of cellular adaptation induced by simultaneous subjection to invertase and *S. meliloti* LPS the dataset was visualised using MapMan. Figure 4 shows a representation of the cells basic metabolic pathways in response to the combined treatment. The overall response shows, e.g., a strong induction of the secondary metabolism, some rearrangements in cell wall metabolism, and induction of several transcripts involved in the ascorbate and glutathione metabolism. In general, the responses observed upon treatment with invertase and rhizobial LPS resembled transcriptional rearrangements followed by elicitation with invertase alone. Nevertheless, the described
response patterns are induced to a lesser extent in the presence of *S. meliloti* LPS. To investigate a possible suppression effect of the rhizobial LPS on the global level, the differences between induction rates for each gene of both datasets were calculated. Genes for which rhizobial LPS had a moderating effect on transcriptional adjustment were characterised by negative values, whereas for genes which were even stronger altered after simultaneous treatment with invertase and *S. meliloti* LPS compared to elicitation with invertase alone the calculated values were positive. In order to analyse the global effects of *S. meliloti* LPS on defense-associated gene expression the resulting values for differences in transcriptional response ratios were visualised by MapMan using a representation of the cells general metabolism (Fig. 5). It has to be emphasised that unlike the diagrams discussed before this representation does not visualise an unprocessed microarray dataset but the calculated differences between two datasets that were compared. The comparison of the two datasets clearly showed, that *S. meliloti* LPS has a moderating effect for nearly all genes that are displayed in Figure 5. This does affect genes which are induced by invertase elicitation as well as genes which are down regulated in response to the pathogenic stimulus. The visualisation shows very few genes, for which the rhizobial LPS seems to have a stimulating effect on defense associated transcriptional rearrangement. A closer examination of these genes showed, that with one exception (TC80744, a gene annotated as “unknown”) the relative response ratios for these transcripts were comparably low in both datasets that were compared. Thus, the calculated positive values for the differences in response ratios could be due to the inaccuracy of the *M* values obtained in the microarray experiments. Of the 323 genes induced 2-fold or more after invertase treatment that were compared to the dataset of cells treated with invertase and *S. meliloti* LPS only 19 transcripts showed a positive value for the differences in relative response ratios. For all other genes the calculated values indicated a moderating effect of rhizobial LPS on elicitor induced transcription activity, i.e., values of differences in relative response ratios were negative. Nevertheless, the datasets obtained by the microarray experiment do not indicate a fixed degree for the suppression of transcriptional reorganisation. Closer examination of nodule-specific genes did confirm the suppression effect of *S. meliloti* LPS on those nodulins that were induced upon invertase treatment (e.g. TC77131 encoding a nodulin 12A precursor). Nevertheless, significant transcriptional rearrangements in response to rhizobial LPS alone could not be observed.

To confirm the moderating effect of rhizobial LPS on elicitor-induced transcriptional regulation we monitored gene induction of 8 genes using real-time RT-PCR. TCs selected for this approach included 6 genes induced upon invertase treatment and 2 genes which were
repressed after elicitation and for which the microarray approach had shown a strong suppression effect mediated by *S. meliloti* LPS. It should be mentioned, that for the chalcone synthase gene (TC 85174) no unique primers could be designed and melting curves indicated the amplification of at least two different transcripts. Therefore, real-time RT-PCR monitored the abundance of a mixture of at least two different CHS genes. For all 6 TCs that were identified via microarray hybridisation to be induced upon elicitation the induction could be confirmed (Fig.6). Furthermore, the induction of the genes was significantly suppressed in cells treated simultaneously with invertase and *S. meliloti* LPS (*p* ≤ 0.05; *t*-test). Regarding the two TCs that were identified to be repressed upon invertase treatment only for TC81805 encoding a MYB-related transcription factor the repression of transcriptional activity could be confirmed. For both of these genes rhizobial LPS did not show any significant moderating effect on transcriptional regulation [data not shown]. Therefore, the suppression effect could only be confirmed for genes that are induced during elicitation although the calculated differences in relative response ratios visualised in Figure 5 clearly indicate that *S. meliloti* LPS has a moderating effect on both induction and repression of genes.

These results show two important characteristics of the *S. meliloti* LPS mediated suppression of defense-associated gene expression: i) Global visualization of the differences in relative transcript levels of cells elicited with invertase and cells concomitantly treated with invertase and *S. meliloti* LPS shows that the lipopolysaccharide has a moderating effect on the transcriptional adaptation of genes induced as well as on those repressed upon elicitation. ii) the suppression effect applies to all kinds of cellular processes indicating an early interference of the LPS with signal transduction pathways.

However, by which means the rhizobial LPS targets the transmission of recognition of a pathogenic signal molecule into plant defense responses remains elusive. A different kind of suppressor molecule of rhizobial origin, the cyclic 1,3-1,6-β-glucans from the soybean symbiont *Bradyrhizobium japonicum* have been shown to succeed in the competition with the β-glucan elicitor of *Phytophthora sojae* for its binding sites (for a review see Mithöfer, 2002). This competitive binding does not only inhibit the production of phytoalexins (Mithöfer et al., 1996) but also earlier defense-associated signalling events, i.e., an increase in cytosolic Ca²⁺ concentration (Mithöfer et al., 1999). However, unlike *S. meliloti* lipopolysaccharides the cyclic β-glucans from *B. japonicum* do not suppress but induce the production of ROI in the host plant.
Another mechanism that could be responsible for the \textit{S. meliloti} LPS mediated suppression of plant defense is the interference with basal elements of the signal transduction pathways responsible for the activation of defense responses. In the case of the phytopathogenic fungus \textit{Mycosphaerella pinodes} it has been shown that a glycopeptide termed supprescin is able to suppress plant defense at different levels including the induction of PAL transcription (Wada et al., 1995). The mechanism of suppression is likely to involve the interference of the supprescin glycopeptide with a plasma membrane ATPase via direct interaction (Kato et al., 1993). Furthermore, the supprescin of \textit{M. pinodes} does interfere with the PI metabolism in the plant plasma membrane that is essential for the activation of defense responses (Shiraishi et al., 1994). It has been shown in studies on pea plasma membranes and epicotyls, that elicitation leads to a rapid and biphasic increase in levels of phosphatidylinositol-4,5-bisphosphate and inositol 1,4,5-triphosphate whereas the addition of the suppressor molecule inhibited this effect (Toyoda et al., 1992; Toyoda et al., 1993). These findings show that the inhibition of compounds of very early signalling cascades is used by pathogens as a comprehensive mechanism of suppression. Another possibility how suppressors could operate in interfering with defense responses is the active recognition of the molecules via specific receptors. This idea has been proposed for the glycopeptide supprescin from \textit{M. pinodes} by Shiraishi et al. (1994), although to date there is no proof for such a mode of action. However, the specific recognition of suppressors via receptor proteins could mediate a fine tuned regulation of defense responses that are switched on or off upon the concomitant recognition of an elicitor and a suppressor. In contrast, the inhibition of early signalling processes necessarily blocks all downstream processes without any possibility of regulation. In case of suppressors of pathogenic origin the presence of a receptor protein without any other function appears unlikely since it would cause an immense evolutionary disadvantage rendering the plant susceptible to pathogens producing the appropriate suppressor molecule. Nevertheless, in case of symbiotic interactions the presence of receptors specific for suppressors of symbiotic origin would enable different kinds of beneficial responses including a fine tuned suppression of plant defense.

Surface polysaccharides act as bacterial signals in pathogenic and symbiotic interactions (Djordjevic et al., 1987). Although to date the role of the rhizobial lipopolysaccharide in the establishment and maintenance of symbiosis could not be elucidated in detail there is evidence for its importance in the interaction. LPS mutants of \textit{Rhizobium leguminosarum} as well as \textit{S. meliloti} show severe defects in the establishment of symbiosis with their host plants.
(Vedam et al., 2004). In some cases induced nodules are small, poorly infected and show signs of plant defense, e.g., accumulation of cell wall material and phenolic compounds and autofluorescence (Perotto et al., 1994; Niehaus et al. 1998). In both studies bacteria were able to induce and colonize infection threads but failed to differentiate after infecting nodule tissue. The death of the rhizobial mutants during the infection process was discussed either to be due to an impairment of the bacterial protection against unfavourable conditions or to the inability to interfere with the host’s defense response. Another mutant study showed, that not only the presence but also the correct modification of LPS is needed for optimal nodulation activity (Keating et al., 2002). Furthermore, purified LPS of the microsymbiont has been shown to promote the infection in case of the Rhizobium leguminosarum bv. trifolii – white clover symbiosis and to cause the production of novel proteins (Dazzo et al., 1991). The principle of the ability of rhizobial LPS to suppress plant defense responses in the host was achieved by demonstrating the suppression effect on the elicitor induced oxidative burst (Albus et al., 2001). Nevertheless, it is known that during the symbiotic interaction a prolonged production of hydrogen peroxide can be detected in the infection threads and the infection zone of the nodule, a process most likely involved in the regulation of nodulation (Santos et al., 2001). The fact that the oxidative burst during the infection process does not lead to abortion of the symbiotic interaction is due to the high activity of antioxidative proteins (Dalton et al., 1998). Therefore, it was a crucial step to demonstrate the suppression of plant defense responses different from the oxidative burst. The present study demonstrates that S. meliloti LPS has the capacity to interfere with the transcriptional response towards pathogenic signalling further emphasizing on the importance of LPS during a successful symbiotic interaction.

Material and Methods

Plant material

Medicago truncatula “Jemalong” cell suspension cultures were obtained and maintained as described by Scheidle et al. (2005).

Cultivation of bacteria and isolation of LPS
S. meliloti wild-type strain 2011 (Casse et al., 1979) was grown on tryptone yeast agar plates supplemented with 0.4% glucose (w/v) at 28°C for 3 d. Bacteria were washed from the plates with 0.9% NaCl (w/v) and centrifuged at 5000 g and 4°C for 20 min. Cells were washed three times with cold H2O, resuspended in H2O and LPS was extracted via the hot phenol-water method (Westphal and Jann, 1965). The water phase was dialysed for 3 d against H2O. Proteins and nucleic acids were removed by treatment with 100 µg mL⁻¹ DNase I (Serva, Heidelberg, Germany), 15 µg mL⁻¹ RNase (Serva, Heidelberg, Germany) and 150 µg mL⁻¹ proteinase K (Roche, Mannheim, Germany). Fractions were dialysed against H2O for 2 d and lyophilised. The lyophilisate was resuspended in H2O and purified by ultracentrifugation at 100 000 g and 4°C for 8 h. Centrifugation was repeated and the pellet resuspended in H2O and lyophilised. For the experiments the LPS was dissolved in H2O and ultrasonicated on ice for 20 min.

**Treatment of plant cell cultures**

For each biological replicate of microarray hybridisations an appropriate number of M. truncatula suspension cell cultures was pooled. A sample of the pooled cells was sieved and 4 x 2 g of cells were removed for oxidative burst measurements. The remaining cell suspensions were realiquoted to sterile 100 mL flasks and incubated under standard cultivation conditions for 4 to 5 h. After the determination of the oxidative burst using the cells removed from the pooled cultures had confirmed responsiveness of the suspension cells, realiquoted cultures were treated with H2O (500 µL), 50 µg mL⁻¹ yeast invertase (Sigma, Munich, Germany), 50 µg mL⁻¹ S. meliloti LPS or a combination of 50 µg mL⁻¹ invertase and 50 µg mL⁻¹ S. meliloti LPS. After treatment for 4 h each cell culture was sieved to remove the cells from the medium and cells were rapidly frozen in liquid nitrogen. Four independent biological replicates were used for subsequent isolation of RNA.

**Oxidative burst measurements**

To confirm responsiveness of the cell suspensions used for microarray hybridisation, 2 g of cells were resuspended in 8 mL of preincubation medium (3% sucrose (w/v) in 4% MS (v/v) (Murashige and Skoog, 1962). Cells were incubated under culture conditions for 3 h. The
oxidative burst was determined using the H$_2$O$_2$-dependent chemiluminescence reaction according to Warm and Laties (1982). After cell cultures were subjected to one of the treatments used in these experiments, 200 µL of the suspension cultured cells were added to 700 µL 50 mM phosphate buffer (pH 7.9) and 100 µL 1.2 mM luminol (Sigma, Munich, Germany). The reaction was started by adding 100 µL of 14 mM potassium-hexacyanate (Fluka, Seelze, Germany). Luminescence was monitored using a Sirius luminometer (Berthold detection systems, Pforzheim, Germany)

**RNA extraction and microarray hybridisation**

Total RNA from suspension cultured cells was extracted using TRI reagent (Sigma, Munich, Germany) and purified using Microcon-30 columns (Millipore, Schwalbach, Germany). 20 µg of RNA were used to synthesize Cy3- and Cy5-labelled cDNA as described by Hohnjec et al. (2005). Microarray analysis was performed using Mt16kOLI1Plus microarrays. The microarray is based on the Mt16kOLI1 microarray described by Hohnjec et al. (2005) but carries an additional set of 384 oligonucleotides. The array definition file of the microarray can be viewed at [http://www.ebi.ac.uk/arrayexpress/](http://www.ebi.ac.uk/arrayexpress/) under accession number A-MEXP-138. Shortly, the microarray is composed of 16470 70-mer oligonucleotide probes representing all TCs of the TIGR *M. truncatula* gene index, release 5.0, and several controls as well as 384 oligomers representing transcription factors and other regulators. Each probe as well as the controls are spotted in two replicates per microarray. Hybridization of targets, image acquisition and analysis of image data were performed in accordance to Hohnjec et al. (2005). Data files were analysed using the EMMA 2.0 software (Dondrup et al., 2003). Lowess normalization was performed using a floor value of 20 and a *t*-test was applied to identify differentially expressed genes.

Microarray analysis was performed using four independent biological replicates per experimental treatment hybridised to four independent microarrays each facilitating two technical replicates. Complete transcriptome profile datasets can be viewed at [http://www.ebi.ac.uk/arrayexpress/](http://www.ebi.ac.uk/arrayexpress/) under accession numbers A-MEXP-???.

**Adaptation of the software tool MapMan to the *M. truncatula* system**
In order to adapt the MapMan software (Thimm et al., 2004; Usadel et al., 2005; http://gabi.rzpd.de/projects/MapMan/) to the *M. truncatula* Mt16kOL11Plus microarray, the 34 major MapMan BINs and their subbins used for the classification of *A. thaliana* genes were transferred to the *M. truncatula* system. Additionally, the subbin 26.31, “misc.nodulins” was added for the nodulins not appearing in Arabidopsis. The predicted proteins encoded by the 16470 TC sequences present on the *M. truncatula* microarray were compared to *A. thaliana* sequences (TAIR release 6.0) using an initial arbitrary E-value cut-off of $1 \times 10^{-10}$. In addition, Interpro domains were identified and used for functional classification. The classification based on the similarity to Arabidopsis proteins was then automatically checked against domains which have been assigned to a MapMan category. Furthermore, Medicago TCs that did not meet the prerequisites for an automatic draft assignment were classified manually according to their respective TIGR annotation (TIGR release 7.0). Finally all draft assignments were corrected manually for potential mistakes.

**RT-PCR**

To validate results obtained by microarray hybridisations, RT-PCR was performed for eight genes using gene-specific primers (Supplemental Table II). Peroxidase-specific Primers (TC 85182) were not unique and led to the amplification of at least two different transcripts. Primers had a calculated Tm of 53 ± 0.4°C and amplificates were not longer than 300 bp. RT-PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Quiagen, Hilden, Germany). 50 ng of DNaseI-treated (Serva, Heidelberg, Germany) total RNA was used in a total volume of 25 µL. Amplification was monitored using the Opticon real-time PCR cycler (MJ Research, Waltham, MA, USA) and quantified via the Opticon Monitor analysis software version 1.05. The program used for amplification and the method for calculation of relative gene expression are described in Hohnjec et al. (2003). For the normalisation of gene expression the constitutively expressed gene encoding an elongation factor 1-alpha (TC85181, TIGR *M. truncatula* Gene Index 7.0) was used as a reference.

**Supplemental Material**

Table S1:
Compilation of relative log2 expression ratios for *M. truncatula* suspension cell cultures treated either with 50 µg mL\(^{-1}\) invertase or 50 µg mL\(^{-1}\) *S. meliloti* LPS or a combination of 50 µg mL\(^{-1}\) invertase and 50 µg mL\(^{-1}\) *S. meliloti* LPS in comparison to water treated control cells.

Table S2:
Primers used for real-time RT-PCR experiments.

Figure S1:
Effect of lipopolysaccharides from *S. meliloti* and *X. campestris* pv. *campestris* on the invertase induced oxidative burst in *M. truncatula* suspension cultured cells. *M. truncatula* cell suspensions were treated with 50 µg mL\(^{-1}\) invertase (closed squares), 50 µg mL\(^{-1}\) invertase + 50 µg mL\(^{-1}\) *X. campestris* pv. *campestris* LPS (lines), 50 µg mL\(^{-1}\) invertase + 50 µg mL\(^{-1}\) *S. meliloti* LPS (closed triangles), 50 µg mL\(^{-1}\) *X. campestris* pv. *campestris* LPS (closed circles), 50 µg mL\(^{-1}\) *S. meliloti* LPS (asterisks) or water as a negative control (closed diamonds). The production of hydrogen peroxide was determined utilizing the luminol based chemiluminescence assay.

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Figures

Figure 1:
Induction and suppression of the oxidative burst in M. truncatula suspension cultured cells upon treatment with invertase and invertase + S. meliloti lipopolysaccharide, respectively. M. truncatula cell suspensions were treated with 50 µg mL⁻¹ invertase (closed squares), 50 µg mL⁻¹ invertase + 50 µg mL⁻¹ S. meliloti LPS (open triangles) or water as a negative control (closed diamonds). The production of hydrogen peroxide was determined utilizing the luminol based chemiluminescence assay. The oxidative burst measured for one of the four biological replicates is shown.

Figure 2:
Classification of the 336 genes found to be at least 2-fold induced in M. truncatula suspension cells upon 4 h treatment with 50 µg mL⁻¹ invertase. The genes classified had to meet the prerequisites \(-1 \leq M \leq 1, p \leq 0.05, A \geq 5.5\) and \(n \geq 6\). A complete list of the classified genes is given in Supplemental Table I. The number of genes allocated to each functional category is indicated, the functional categories are defined.

Figure 3:
Changes in transcript levels in invertase treated M. truncatula suspension cell cultures. Cells were elicited for 4 h using 50 µg mL⁻¹ invertase. Displayed are genes associated with the general metabolism (A) and with cellular responses (B). Red and blue displayed signals represent a decrease and increase in transcript abundance, respectively, relative to water treated control cells. The scale used for colouration of the signals (log2 ratios) is presented. The picture can be viewed and descriptions for each signal accessed under http://gabi.rzpd.de/.
Figure 4:
Changes in transcript levels in *M. truncatula* suspension cell cultures treated simultaneously with invertase and *S. meliloti* LPS relative to water treated control cells. Cell cultures were subjected for four hours to treatment with 50 µg mL⁻¹ invertase and *S. meliloti* LPS, each. Displayed are genes associated with the general metabolism. Red and blue displayed signals represent a decrease and increase in relative transcript abundance respectively. The scale used for colouration of the signals (log2 ratios) is presented. The picture can be viewed and descriptions for each signal accessed under http://gabi.rzpd.de/.

Figure 5:
Visualisation of the suppression effect of *S. meliloti* LPS on defense-associated gene transcription in *M. truncatula* cell cultures. Represented are the differences between the relative response ratios of invertase (50 µg mL⁻¹) treated suspension cells and cell cultures treated with 50 µg mL⁻¹ invertase and 50 µg mL⁻¹ *S. meliloti* LPS, each. Red and blue displayed signals represent a moderating or increasing effect of the rhizobial LPS on elicitation induced transcriptomic reorganisation, respectively. The scale used for colouration of the signals (differences of the log2 ratios of the compared datasets) is presented. The picture can be viewed and descriptions for each signal accessed under http://gabi.rzpd.de/.

Figure 6:
Relative suppression of invertase induced gene regulation in *M. truncatula* mediated by *S. meliloti* LPS. The regulation of 6 genes (see material and methods) that were confirmed via microarray hybridization to be regulated upon invertase treatment was monitored via real-time RT-PCR. Transcript abundances of cells treated with 50 µg mL⁻¹ invertase or with 50 µg mL⁻¹ invertase and 50 µg mL⁻¹ *S. meliloti* LPS, each, were determined relative to water treated control cells and normalized against *mtTefα* gene expression. Black and white columns represent relative induction rates of cell cultures treated with invertase or invertase and *S. meliloti* LPS, respectively. Experiments were performed in four biological and two technical replicates. Presented here are the arithmetic mean and the standard error of the data. COMT, caffeic acid O-methyltransferase; CHS, chalcone synthase; path. rel. transcr. factor, pathogenesis related transcription factor; PAL phenylalanine ammonia lyase; IFR isoflavone reductase.
Table I: Overview of the effect of invertase treatment on *M. truncatula* suspension cells

Listed are the 20 most strongly induced and repressed genes obtained from transcriptomic profiling of invertase treated cells in comparison to water treated cultures. Suspension cultured *M. truncatula* cells were treated for 4 h with either 50 µg mL⁻¹ invertase or water as a reference. Oligo ID, identifier of *M. truncatula* 70-mer oligonucleotides. TIGR ID, identifier in the TIGR *M. truncatula* gene index 7.0. Annotation, annotation in the TIGR *M. truncatula* gene index 7.0. INV and INV+LPS<sub>Sm</sub>, log2 expression ratios in invertase and invertase + *S. meliloti* LPS treated suspension cells in comparison to a water treated control.

| Oligo ID | TIGR ID | Annotation                                     | INV  | INV + LPS<sub>Sm</sub> |
|----------|---------|-----------------------------------------------|------|------------------------|
| MT014241 | TC85478 | Isoflavone reductase                          | 3.970| 3.029                  |
| MT014270 | TC85521 | Chalcone reductase                            | 3.541| 1.912                  |
| MT015080 | TC85174 | Chalcone synthase 4                           | 3.457| 2.094                  |
| MT000150 | TC85477 | Isoflavone reductase                          | 3.373| 2.313                  |
| MT015632 | TC87485 | Germin-like protein                           | 3.361| 2.410                  |
| MT015147 | TC76769 | Chalcone synthase 4                           | 3.352| 2.234                  |
| MT008395 | TC78488 | Germin-like protein                           | 3.290| 1.951                  |
| MT014469 | TC86777 | Cyanogenic Beta-Glucosidase                   | 3.289| 1.967                  |
| MT015072 | TC85146 | Chalcone synthase 2                           | 3.271| 2.018                  |
| MT015146 | TC85138 | Chalcone synthase 8                           | 3.260| 2.059                  |
| MT015144 | TC76770 | Chain A Chalcone Synthase                     | 3.243| 2.064                  |
| MT015149 | TC76765 | Putative chalcone synthase                    | 3.213| 1.932                  |
| MT015480 | TC77750 | Chitinase                                     | 3.084| 2.059                  |
| MT015145 | TC76767 | Chalcone synthase 2                           | 3.045| 1.853                  |
| MT015083 | TC85174 | Chalcone synthase 4                           | 3.042| 1.885                  |
| MT015076 | TC85150 | Chalcone synthase 9                           | 2.795| 1.802                  |
| MT000333 | TC85502 | Phenyllalanine ammonia-lyase                  | 2.762| 1.986                  |
| MT000259 | TC76871 | Hypothetical protein                          | 2.723| 1.529                  |
| MT007784 | TC86455 | Isoflavone reductase                          | 2.671| 1.415                  |
| MT000494 | TC86191 | Hypothetical protein                          | 2.634| 1.477                  |
| MT000936 | TC78069 | Oligo-TC                                      | -2.539| -2.715                 |
| MT007017 | TC85263 | Putative plasma membrane intrinsic protein    | -1.786| -1.283                 |
| MT007036 | TC76601 | Aquaporin-like protein PIP2                   | -1.751| -0.993                 |
| MT008134 | TC78229 | Alpha-xylosidase precursor                    | -1.678| -1.427                 |
| MT007395 | TC85871 | Pectate lyase                                 | -1.669| -0.959                 |
| MT014301 | TC76880 | Endoxyloglucan transferase                    | -1.662| -1.131                 |
| MT016122 | Autointerpro | MYB related protein...<sup>a</sup> | -1.563| -0.681                 |
| MT009717 | TC79773 | AtHVA22a 65476-64429                          | -1.472| -0.863                 |
| MT013556 | TC80306 | Hypothetical protein                          | -1.462| -0.784                 |
| MT015781 | TC81234 | Unknown                                       | -1.460| -0.329                 |
| MT010917 | TC82525 | Unknown                                       | -1.442| -0.517                 |
| MT014300 | BQ136812| Xyloglucan endotransglycosylase               | -1.372| -0.902                 |
| MT011472 | TC82617 | AT5g46260/MSJ110                              | -1.315| -1.191                 |
| MT002409 | TC88503 | Endo-beta-1 4-gluanacase                      | -1.281| -0.804                 |
| MT001311 | TC87188 | At2g17230/T23A1.9                             | -1.277| -0.929                 |
| MT016449 | Autointerpro | Leucine rich repeat containing protein<sup>b</sup> | -1.266| -1.093                 |
| MT015278 | TC76902 | MtN5                                          | -1.234| -1.031                 |
| MT001226 | TC87263 | Unknown                                       | -1.233| -0.880                 |
| MT007159 | TC85575 | Protein F3M18.16                              | -1.230| -1.083                 |
| MT013339 | TC84195 | Putative beta-galactosidase                   | -1.218| -0.838                 |
induction rate relative to water treated control

- COMT
- CHS
- Peroxidase
- Path to terpene biosynthesis
- PAL
- IFR