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Identification of genes differentially expressed in *Mikania micrantha* during *Cuscuta campestris* infection by suppression subtractive hybridization

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Summary

The influence of *Cuscuta campestris* on its host *Mikania micrantha* has been studied with respect to biomass accumulation, physiology and ecology. Molecular events of this parasitic plant–plant interaction are poorly understood, however. In this study, we identified novel genes from *M. micrantha* induced by *C. campestris* infection. Genes expressed upon parasitization by *C. campestris* at early post-penetration stages were investigated by construction and characterization of subtracted cDNA libraries from shoots and stems of *M. micrantha*. Three hundred and three presumably up-regulated expressed sequence tags (ESTs) were identified and classified in functional categories, such as ''metabolism'', ''cell defence and stress'', ''transcription factor'', ''signal transduction'', ''transportation'' and ''photosynthesis''. In shoots and stems of infected *M. micrantha*, genes associated with defence responses and cell wall modifications were induced, confirming similar data from other parasitic plant–plant interactions. However, gene expression profiles in infected shoots and stems were found to be different. Compared to infected shoots, more genes induced in response to biotic and abiotic stress factors were identified in infected stems. Furthermore, database comparisons revealed a notable number of *M. micrantha* ESTs that matched genes with unknown function. Expression analysis by quantitative real-time RT-PCR of 21 genes (from different

Abbreviations: AGP, arabinogalactan protein; CAX, calcium exchanger; cDNA, complementary DNA; CUC, cup-shaped cotyledon; CDPK, calcium-dependent protein kinase; dpi, days post-infection; EREBF, ethylene-responsive element binding factor; EST, expressed sequence tag; IPTG, isopropyl-β-D-thiogalactopyranoside; JMT, jasmonic acid carboxyl methyltransferase; LB, luria broth; Leaqp2, aquaporin; Lexth1, xyloglucan endotransglycosylase/hydrolase; MBP, myrosinase binding protein; NAC, NAM, ATAF1/2, CUC2; NAM, no apical meristem; PCR, polymerase chain reaction; PR, pathogenesis-related; RT-PCR, reverse transcription polymerase chain reaction; SAM, S-adenosyl-L-methionine; SAMS, S-adenosylmethionine synthase; SSH, suppression subtractive hybridization; TMT, tonoplast monosaccharide transporter; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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functional categories) showed significantly increased levels for 13 transcripts in response to *C. campestris* infection. In conclusion, this study provides an overview of genes from parasitized *M. micrantha* at early post-penetration stages. The acquired data form the basis for a molecular understanding of host reactions in response to parasitic plants.

Introduction

Cuscuta spp. plants, belonging to the Convolvulaceae family, are root- and leafless holoparasites on various host plants. *Cuscuta campestris* (field dodder) is the most widespread dodder in the world (Dawson et al., 1994). *C. campestris* develops haustoria, which penetrate and contact the host vascular tissue to absorb water, carbohydrates and minerals. After penetration of the host tissue by haustorium cells, a ''parasite fissure'' is formed in the host stem (Dawson et al., 1994).

Mikania micrantha, one of the 100 worst invasive alien species in the world (Lowe et al., 2001), is a perennial vine belonging to the Asteraceae family. The species is native to Central and South America, where it is a weed of minor importance (Holm et al., 1977; Wirjahar, 1976). In recent decades, *M. micrantha* has caused severe damage to many ecosystems in southern China (Zhang et al., 2004).

A previous study has shown that *C. campestris* can suppress the growth of *M. micrantha* (Parker, 1972). Recently, the effects of *C. campestris* on *M. micrantha* in southern China have been studied with respect to biomass accumulation, physiology and ecology. For example, pot trials showed that *C. campestris* significantly reduced total biomass, changed biomass allocation patterns, completely inhibited flowering, and also reduced photosynthesis of the parasitized *M. micrantha* plants (Shen et al., 2005, 2007). Furthermore, field trials showed that *C. campestris* significantly reduced growth of *M. micrantha* (Lian et al., 2006). These results suggested that *C. campestris* could be used as an effective way to control *M. micrantha* (Lian et al., 2006; Shen et al., 2005, 2007).

In a preliminary study, we observed the haustorium development of *C. campestris* in stems of *M. micrantha* by conventional microscopy (Supplementary Figure 1). The first visible haustoria were formed in host stems after 2 days post-infection (dpi) (Figure 1A). At this stage, the haustoria did not connect to the vascular tissues of the host plant until the next 2 d (Figure 1B). At 7 dpi, haustoria connected to the host xylem at high frequency (Figure 1C and D). In this study, the early post-penetration stages at 2 dpi (formation of first haustoria) and at 7 dpi (many haustoria start to connect to the host xylem) were chosen for identification of *M. micrantha* genes induced upon infection by *C. campestris*.

Work on gene expression in parasitized plants has been performed with model host plants such as *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*). Transcriptional changes in tobacco have been studied in response to *Orobanche aegyptiaca* infection (Griffitts et al., 2004; Joel and Portnoy, 1998; Westwood et al., 1998). Target-gene expression studies revealed the involvement of several *A. thaliana* genes upon *O. ramosa* infection (Vieira Dos Santos et al., 2003a). Application of differential display approaches also identified various induced genes in roots of the non-host marigold (*Tagetes erecta*) in response to *Striga asiatica* (Gowda et al., 1999) and in dodder-infected alfalfa (*Medicago sativa*) plants (Borsics and Lados, 2001, 2002). Using a suppression subtractive hybridization (SSH) strategy (Diatchenko et al., 1996), three tomato genes, *Lexth1* (Albert et al., 2004), *attAGP* (Albert et al., 2006) and *Leaqp2* (Werner et al., 2001) were found to be induced at infection sites of *C. reflexa*. These genes encode a xyloglucan endotransglycosylase/hydrolase, an arabinogalactan protein (AGP) and an aquaporin, respectively. Moreover, other differentially expressed genes in parasitized plants were identified by using SMART technology (Clontech, USA) and the SSH method (Die et al., 2007; Letousey et al., 2007; Vieira Dos Santos et al., 2003b). Data on expression of *M. micrantha* genes during *C. campestris* infection has not been acquired so far, however.

The purpose of the present study was to identify genes in shoots and stems of *M. micrantha*, which are induced in upon infection with *C. campestris* at early post-penetration stages. Genes differentially expressed were identified with SMART technology and the SSH method. The isolated expressed sequence tag (EST) clones were sequenced and compared with sequence databases. The obtained collection of identified genes provides an important genomic resource for future molecular analysis of

the infection process in the interaction between *M. micrantha* and *C. campestris*.

Materials and methods

Plant materials and growth conditions

Whole *Mikania micrantha* H. B. K. (bittervine or climbing hempweed) plants were collected from a population in Zhuhai, Guangdong province, China. Two-node segments, similar in size, were planted into pots (18 cm in height, 22 cm in upper diameter and 15 cm in lower diameter), which were filled with a mixture of humus soil, perlite and sand (3:1:1 in volume). Plants were maintained under greenhouse conditions for 2 months ($25 \pm 1\,^{\circ}C$; $75 \pm 10\%$ relative humidity; 300 μmol photons $m^{-2} s^{-1}$ light intensity with 16 h light–8 h dark photoperiod). A bamboo cane (about 1.5 m in length) was placed vertically in each pot, on which *M. micrantha* climbed. *Cuscuta campestris* Yuncker (field dodder) was cultivated on *Wedelia trilobata* (L.) Hitch. under the same growth conditions as for *M. micrantha* plants. *W. trilobata* was chosen as the ''donor host'' for *C. campestris* cultivation. *W. trilobata* displayed a high tolerance to *C. campestris* infection and remained alive for a long time.

Inoculation with *C. campestris*

For infection of *M. micrantha* plants, *C. campestris* shoots (about 20 cm in length) were coiled around the green stems of 2-month-old *M. micrantha* plants. This time point was considered as the initial time of the infection process. Six *M. micrantha* plants were infected and six other plants were served as non-infected controls. Infected and non-infected *M. micrantha* plants were watered with sterile distilled water once a day and kept under the growth conditions as described above.

Collection of samples

Three shoots of infected and non-infected *M. micrantha* plants were collected individually at 2 dpi (formation of first visible haustoria). Furthermore, three infected stem parts close to the parasite attachment site of *C. campestris* (0.5 cm) and similar stem parts from non-infected *M. micrantha* plants were harvested individually at 7 dpi (many haustoria started to connect to the host xylem). These four collected samples were washed with distilled water, dried with filter paper, immediately frozen in liquid nitrogen and stored at $-80\,^{\circ}C$.

RNA extraction and amplification by SMART technology

Total RNA was isolated from shoots according to the method described by Ding et al. (2008). In order to minimize variation in gene expression among individual plants, samples (1.0 g per plant) were taken from different pools of three infected and non-infected shoots. The quantity and quality of the isolated total RNA was checked on denaturing agarose gels and by measuring the absorbance at 230, 260 and 280 nm on a UV spectrophotometer. Isolation of total RNA from infected and non-infected stems was prepared in a similar way.

Total RNA (1.0 μg per sample) was then reverse-transcribed and amplified to produce SMART complementary DNA (cDNA) using the SMART™ PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA) according to the user's manual.

Construction of subtracted cDNA libraries

Two forward subtracted cDNA libraries in terms of different infection days and tissues were constructed using the PCR-select cDNA Subtraction Kit (Clontech). For SSH library 1, the tester and the driver cDNA population were generated from total RNA extracted from shoots of infected and non-infected *M. micrantha* plants at 2 dpi, respectively. For SSH library 2, the tester and the driver cDNA population were generated from total RNA extracted from stem parts of infected *M. micrantha* close to the attachment site of *C. campestris* (0.5 cm) at 7 dpi and from similar stem parts from non-infected *M. micrantha* plants, respectively. The subtracted cDNA fragments were directly cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and transformed into competent cells of *Escherichia coli* DH5α (Dingguo, Beijing, China). Transformed cells were grown on standard LB/ampicillin/X-gal/IPTG plates at $37\,^{\circ}C$ for blue-white colony screening.

cDNA sequencing and data analysis

Plasmid DNA isolation was performed for a total of 250 (library 1) and 258 (library 2) white clones randomly picked from plates. Plasmids containing cDNA fragments were sequenced with either T7 primer (5′TAATACGACTCACTATAGGG3′) or SP6 primer (5′AGCTATTTAGGTGACACTATAG3′) on an ABI 3700 sequencer (Applied Biosystems). The

sequences were trimmed to eliminate vector sequences, SMART-cDNA primers and SSH-adaptor sequences. The inserted sequences were manually checked for similarities in the GenBank database using the Blastx program (Altschul et al., 1997) on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST). The ESTs with significant database matches (*E*-value $\leqslant$1e$-$5) were classified into functional categories by according to the Gene Ontology database (http://www.geneontology.org) and deposited into the GenBank database under the accession numbers indicated below.

Data validation by quantitative real-time RT-PCR

Real-time RT-PCR reactions were performed in optical reaction tubes using an iCycler iQ5 apparatus (BioRad, Hercules, CA, USA). The RNA samples were identical to those described above. Total RNA of each sample was treated with RNase-free Dnase I (TaKaRa) to remove DNA contamination. cDNA of each sample was synthesized using the PrimeScript™ Reagent Kit (TaKaRa) according to the manufacturer's protocol. The gene-specific primers were designed by Prime Primer 5 (Table 4). Quantitative real-time PCR was carried out with the real-time SYBR green kit (TaKaRa) in a final volume of 25 μL, including 0.2 μM of each primer, 12.5 μL SYBR Premix (2 ×), 2 μL of diluted first strand cDNA, and sterile distilled water. The cycling conditions were as follows: preheating for 1 min at 95 °C followed by 40 cycles (denaturing for 10 s at 95 °C, annealing for 15 s at 59 °C, extension for 15 s at 72 °C, and data acquisition at 81 °C). At the end of the amplification experiment, a melting curve was realized between 55 and 95 °C by steps of 0.5 °C, to ensure the signal corresponded to a single PCR product. In this experiment, the plasmids with each gene were used to prepare standard curves and each gene quantity was determined from the relative standard curves. Only amplifications of primer pairs with a single polymerase chain reaction (PCR) product, and a determination coefficient (R^2) of the standard curve equal or superior to 0.99 were considered valuable. Quantification of each gene was assessed using relative standard curves. A relative expression ratio was calculated as the ratio of normalized gene expression of the target gene against *actin* (a constitutively expressed control gene of *M. micrantha*; GenBank acc. no. EY456955) as previously described by Lu et al. (2007). Each gene expression analysis was carried out with three experimental replicates followed by a Student's *t*-test to estimate whether expression values for infected plants and non-infected plants were significantly different.

Results

SSH library of *M. micrantha* shoots infected by *C. campestris*

RNA from *M. micrantha* shoots infected by *C. campestris* and non-infected control shoots was used to construct the subtracted cDNA library 1. A total of 250 clones were randomly chosen from the cDNA library enriched for genes expressed upon parasitization by *C. campestris*. Sequencing showed that the size of the inserts varied from 150 to 1100 bp. Database comparisons revealed that 178 non-redundant ESTs exhibited significant homology to plant genes. Based on their putative functions, the ESTs were annotated and classified into 10 categories as follows: ''metabolism'', ''cell defence and stress'', ''transcription factor'', ''signal transduction'', ''transportation'', ''protein synthesis and modification'', ''photosynthesis'', ''cell structure and component'', ''energy'' and ''unknown proteins'' (Table 1). The ''metabolism'' category (consisting of six sub-classes: ''amino acid'', ''C-compound'', ''nucleotide'', ''lipid and fatty acid'', ''phosphate'' and ''secondary metabolism'') contained 31 distinct deduced proteins from 48 ESTs, which accounted for 27.0% (48/178) of the classified ESTs. The category ''protein synthesis and modification'' contained 20 distinct proteins deduced from 23 ESTs, which accounted for 12.9% of the classified ESTs. Other categories were as follows: ''cell structure and component'' (16 distinct proteins; 20 ESTs; 11.2%), ''signal transduction'' (12 distinct proteins; 16 ESTs; 9.0%), ''cell defence and stress'' (10 distinct proteins, 12 ESTs; 6.7%), ''photosynthesis'' (10 distinct proteins; 10 ESTs; 5.6%), ''transportation'' (7 distinct proteins; 8 ESTs; 4.5%), ''energy'' (4 distinct proteins; 7 ESTs; 3.9%), ''transcription factor'' (5 distinct proteins; 5 ESTs; 2.8%) and ''unknown proteins'' (29 ESTs; 16.3%). Some of the EST sequences have been deposited in GenBank under the accession numbers shown in Table 2.

SSH library of *M. micrantha* stems infected by *C. campestris*

The subtracted cDNA library 2 was constructed with RNA from *M. micrantha* stems parasitized by *C. campestris* and non-infected control stems. A total of 258 clones, with insert sizes ranging

Table 1. Functional categorization of *C. campestris*-induced ESTs in *M. micrantha*.

Functional categorization	Library 1, number of ESTs	Ratio (%)	Library 2, number of ESTs	Ratio (%)
Metabolism	48	26.96	28	22.40
Amino acid	8	4.49	1	0.80
C-compound	21	11.79	12	9.60
Nucleotide	8	4.49	6	4.80
Lipid and fatty acid	3	1.68	5	4.00
Phosphate	3	1.68	2	1.60
Secondary	5	2.80	2	1.60
Cell defence and stress	12	6.74	19	15.20
Transcription factor	5	2.80	5	4.00
Signal transduction	16	8.98	10	8.00
Transportation	8	4.49	5	4.00
Protein synthesis and modification	23	12.92	15	12.00
Photosynthesis	10	5.61	8	6.40
Cell structure and component	20	11.23	8	6.40
Energy	7	3.93	5	4.00
Unknown protein	29	16.29	22	17.60
Total	178	100	125	100

from 200 to 1000 bp, were randomly selected, sequenced and categorized according to the same procedure as described above for the library 1. After editing, 125 ESTs showed significant homology to plant genes. Twenty-seven distinct deduced proteins deduced from 28 ESTs belonged to the ''metabolism'' category and accounted for 22.4% (28/125) of the classified ESTs. The category ''cell defence and stress'' consisted of 14 distinct proteins deduced from 19 ESTs, which accounted for 15.2% of the classified ESTs. Other categories were as follows: ''protein synthesis and modification'' (13 distinct proteins; 15 ESTs; 12.0%), ''signal transduction'' (6 distinct proteins; 10 ESTs; 8.0%), ''cell structure and component'' (5 distinct proteins; 8 ESTs; 6.4%), ''photosynthesis'' (7 distinct proteins; 8 ESTs; 6.4%), ''transcription factor'' (5 distinct proteins; 5 ESTs; 4.0%), ''transportation'' (4 distinct proteins; 5 ESTs; 4.0%), ''energy'' (4 distinct proteins; 5 ESTs; 4.0%), and ''unknown proteins'' (22 ESTs; 17.6%). Some of the EST sequences have been deposited in GenBank under the accession numbers shown in Table 3.

By comparing the results of the two subtracted cDNA libraries, only 10 ESTs were found in common for both tissues, which was only 3.3% (10/303). These findings suggest induction of specific genes in shoots and stems of infected *M. micrantha* plants and that a large proportion of the identified ESTs display a different expression pattern, which may reflect tissue-specific differences related to biotic stress response mechanisms. In other words, the observed differences in gene expression are likely the result of an innate difference between shoot and stem tissues. This is reminiscent to gene expression in different strawberry tissues upon fungal infection by *Colletotrichum acutatum* (Casado-Díaz et al., 2006).

Gene expression by real-time reverse transcription polymerase chain reaction (RT-PCR)

Quantitative real-time RT-PCR was used to experimentally verify the pattern of 21 putative *C. campestris*-induced *M. micrantha* genes, which belonged to various functional categories (11 ESTs from library 1 and 10 ESTs from library 2). After real-time RT-PCR, gene induction levels were estimated on the basis of three experimental replicates. Six out of 11 genes (54.5%) were found to be significantly up-regulated in infected shoots and calculated average ratios (values from infected shoots over non-infected shoots) showed a more than 3-fold difference (Table 5). Seven out of 10 stem genes (70.0%) exhibited a more than 1.95-fold increase of accumulated transcripts in response to *C. campestris* infection. In infected stem tissue, the most strongly induced gene (6-fold induction) was *glycolate oxidase* (S152), sharing 91.0% identity to *glycolate oxidase* from *A. thaliana*. Transcript levels of a related *glycolate oxidase* gene were also found to be up-regulated by salt stress in *Populus euphratica* plants (Gu et al., 2004).

Table 2. Genes differentially expressed in *M. micrantha* shoots in response to *C. campestris* infection.

Clone no.	GenBank Acc. no.	Length (bp)	Related sequences (GenBank Acc. no.)	*E*-value
Amino acid metabolism				
St59[a]	EY202292	605	At S-adenosylmethionine synthase 3 (NM_112618.2)	1e−94
St174	EY202407	335	At methionine synthase 2 (NM_111249.4)	2e−35
St177	EY202410	724	At cobalamin-independent methionine synthase (NM_121798.3)	6e−126
C-compound metabolism				
St15[a]	EY202248	508	At curculin-like lectin family protein (NM_106534.1)	8e−38
St186	EY202419	334	At putative mannose-6-phosphate reductase (NM_127697.2)	1e−23
Nucleotide metabolism				
St94	EY202327	729	*Zea mays* DNA cytosine methyltransferase MET2a (NM_001111508.1)	2e−81
St171	EY202404	308	At CID11; RNA binding (NM_001036054.1)	7e−47
Lipid, fatty acid metabolism				
St64	EY202297	642	At fatty acid desaturase 5 (NM_112455.3)	1e−54
St99	EY202332	476	At protease inhibitor/lipid transfer protein family protein (NM_112712.1)	2e−19
Phosphate metabolism				
St23	EY202256	328	At phosphatidic acid phosphatase family protein (NM_115711.3)	3e−48
Secondary metabolism				
St1	EY202234	918	At P450 reductase2 (NM_179141.2)	3e−91
St48	EY202281	637	At cytochrome P450 (NM_119345.2)	1e−40
St62	EY202295	554	At terpene synthase (NM_123830.2)	3e−15
St140	EY202373	510	At prenylated rab acceptor (PRA1) family protein (NM_120620.2)	1e−31
Cell defence and stress				
St30[a]	EY202263	787	At thiol-disulfide exchange intermediate (NM_180903.2)	3e−95
St47[a]	EY202280	339	At copper/zinc superoxide dismutase (NM_100757.3)	6e−38
St54[a]	EY202287	673	At glycine hydroxymethyltransferase (NM_119954.3)	9e−73
St55[a]	EY202288	718	At auxin signaling F-BOX 2 (NM_116163.3)	1e−77
St74	EY202307	577	At F-box family protein (NM_124377.2)	1e−61
St95[a]	EY202328	714	At chitinase (NM_100466.4)	5e−90
Transcription factor				
St89	EY202322	517	At kow transcription factor family (NM_116904.3)	5e−45
St124	EY202357	406	At myb family transcription factor (NM_201656.2)	1e−10
Signal transduction				
St8	EY202357	287	At MAP kinase kinase (NM_123408.2)	1e−40
St12	EY202245	781	At kinase related (NM_102676.3)	4e−60
St37[a]	EY202270	535	At calcium-dependent protein kinase 2 (NM_103271.3)	3e−82
Transportation				
St102[a]	EY202335	726	At tonoplast monosaccharide transporter 2 (NM_179234.1)	3e−65
St134	EY202367	449	At delta tonoplast integral protein (NM_112495.3)	1e−32
Protein synthesis, modification				
St2	EY202235	228	At polyubiquitin 10 (NM_178968.1)	2e−35

Table 2. (*continued*)

Clone no.	GenBank Acc. no.	Length (bp)	Related sequences (GenBank Acc. no.)	*E*-value
Photosynthesis				
St33	EY202266	616	At photosystem II light harvesting complex gene 1.4 (NM_128994.3)	3e−107
St39	EY202272	430	At ribulose bisphosphate carboxylase small chain 1B (NM_123204.3)	4e−53
St156	EY202389	298	At photosystem I subunit L (NM_117349.2)	5e−19
Cell structure, component				
St53[a]	EY202286	346	At thioredoxin family protein (NM_001123776.1)	3e−17
St83[a]	EY202286	642	At fasciclin-like arabinogalactan protein 11 (NM_120395.2)	1e−63
St109	EY202342	318	At beta-galactosidase (NM_179175.2)	2e−40
St147	EY202380	253	At polygalacturonase (NM_102224.3)	2e−33
Energy				
St19	EY202252	455	At putative NADH-ubiquinone oxidoreductase (NM_120938.3)	2e−34

[a]Clones selected for further real-time RT-PCR analysis are marked with an asterisk.

Discussion

In this study, we constructed two subtracted cDNA libraries enriched for *C. campestris*-induced genes in shoots and stems of *M. micrantha*. The libraries reflect the host response to *C. campestris* infection at early post-penetration stages (Table 1). A number of *M. micrantha* ESTs presented in this work are homologous to infection-related genes in various host plants parasitized by *Orobanche*, *Cuscuta* and *Striga*, which have been identified by a targeted gene approach (Griffitts et al., 2004; Joel and Portnoy, 1998; Vieira Dos Santos et al., 2003a), differential display (Borsics and Lados, 2001, 2002), as well as SMART technology combined with the SSH method (Die et al., 2007; Letousey et al., 2007; Vieira Dos Santos et al., 2003b). Other ESTs identified in this study show sequence similarities with genes encoding transcription factors, signal transduction proteins, and proteins related to transport and metabolic processes. Furthermore, real-time RT-PCR has been used in this work to study the expression of 21 randomly selected genes (with putative functions) derived from the sequenced ESTs. Among these genes, 12 genes (57%) were found to be up-regulated at least 3-fold in response to *C. campestris* infection. This is comparable to a SSH library from *Medicago truncatula* roots, where 55% of selected host genes were up-regulated at least 1.7-fold upon infection by *O. crenata* (Die et al., 2007). These results point to the validity of using a combination of SMART technology, SSH, and real-time RT-PCR to identify host genes in plant–plant interactions. Selected *M. micrantha* genes possibly related to *C. campestris* infection will be discussed in the following sections.

Genes involved in metabolism

Expression of various *M. micrantha* genes involved in metabolism and biosynthesis of carbohydrates, nitrogen and fatty acids was induced during *C. campestris* infection at early post-penetration stages. S-adenosylmethionine synthase (SAMS) catalyses L-methionine and ATP to form S-adenosyl-L-methionine (SAM), which is a precursor in the biosynthesis of ethylene. SAMS plays a regulatory role in synthesis of methionine and other aspartate-derived amino acids (Tabor and Tabor, 1984). A function for SAMS in host plants during parasitic plant infection has not been reported so far. The significant up-regulation of *SAMS* transcripts in parasitized *M. micrantha* suggests that SAMS may play an important role in ethylene biosynthesis during *C. campestris* infection. In addition, we also found slightly elevated (non-significant) levels of transcripts encoding curculin-like lectin, carbon–nitrogen hydrolase and fatty acid dehydrogenase. Expression of these genes will be investigated in future studies.

Genes related to phytohormones

Various genes related to the phytohormones ethylene, auxin and jasmonate seem to be induced in stems of parasitized *M. micrantha*. This category includes genes encoding a putative

Table 3. Genes differentially expressed in *M. micrantha* stems in response to *C. campestris* parasitization.

Clone no.	GenBank Acc. no.	Length (bp)	Related sequences (GenBank Acc. no.)	E-value
Amino acid metabolism				
S32	EY274737	774	At L-aspartate oxidase (NP_568304.1)	5e−86
C−compound metabolism				
S18[a]	EY274723	582	At carbon–nitrogen hydrolase protein (NM_121242.4)	3e−51
S106	EY274811	578	At aldose 1-epimerase family protein (NM_121454.2)	2e−36
Nucleotide metabolism				
S69	EY274774	620	At nucleic acid binding (NM_102665.3)	6e−44
Lipid, fatty acid metabolism				
S12	EY274717	453	At lipid transfer protein (NM_111711.3)	2e−10
S56[a]	EY274761	625	At delta12-fatty acid dehydrogenase (NM_112047.3)	1e−60
Phosphate metabolism				
S162	EY274867	469	At inorganic pyrophosphatase protein (NM_121002.3)	2e−39
Secondary metabolism				
S143	EY274848	349	At dienelactone hydrolase protein (NM_103237.3)	8e−16
Cell defence and stress				
S55	EY274760	242	At putative ethylene-responsive element binding protein (NM_001084343.1)	2e−07
S61[a]	EY274766	668	At putative auxin/aluminum-responsive protein (NM_121919.3)	7e−74
S83[a]	EY274788	936	At NHL1 (NDR1/HIN1-like1) (NM_111998.2)	5e−26
S98	EY274803	496	At jasmonate-*O*-methyltransferase (JMT) (NM_101820.3)	2e−39
S120[a]	EY274825	667	At putative peroxidase (NM_120616.2)	2e−62
S152[a]	EY274857	703	At glycolate oxidase (NM_001084685.1)	4e−15
S153	EY274858	741	At universal stress protein (NM_105501.3)	3e−37
Transcription factor				
S53[a]	EY274758	519	At ATAF1 transcription factor (NM_100054.2)	5e−45
S174[a]	EY274879	282	At NAP transcription factor (NM_105616.3)	3e−30
Signal transduction				
S50	EY274755	707	At calcium ion binding (NM_114249.3)	2e−79
S114	EY274819	592	At ARA6, GTP binding (NM_115341.2)	1e−81
Transportation				
S91[a]	EY274796	850	At calcium exchanger 5 (NM_104449.3)	2e−105
S147	EY274852	282	At tonoplast intrinsic protein (NM_129238.3)	1e−38
Photosynthesis				
S47	EY274752	905	At photosystem I light harvesting complex gene 3 (NM_104833.2)	1e−122
Cell structure, component				
S179[a]	EY274884	526	At expansin A10 (NM_001084130.1)	8e−32

Table 3. (*continued*)

Clone no.	GenBank Acc. no.	Length (bp)	Related sequences (GenBank Acc. no.)	E-value
Energy				
S88	EY274793	413	At NADH dehydrogenase subunit 2 (NC_008285.1)	7e−23
S171	EY274876	520	At short chain dehydrogenase/reductase protein (NM_118472.3)	1e−23

[a]Clones selected for further real-time PCR analysis are marked with an asterisk.

Table 4. Primer sequences used for quantitative real-time RT-PCR.

Clone ID	Forward primer (5′→3′)	Reverse primer (5′→3′)	Product length (bp)
S18	CGGCGAATCTCACAACTCT	AGCATAAACAGGGAAACTA	431
S53	GTCCTGAACAATCGGTCCAAA	TTGTAACGGGGAGAGCTGAAA	119
S56	CCACCATGCCAACACCAA	CAAAGGAAACCCGAGAACGA	145
S61	TCACTTGCTTCATTCCCTCAA	TTTGCTCCCAGTATCTCTTCCTC	125
S83	CCTCATCTTAGCCTTCCTG	TGTGATTTGCTGGTTCTTG	238
S91	GGAAATGCAGCAGAACATGC	TCCATCCAACCACCACACA	136
S120	GCATCTACAACGAGAACAACATCAA	CGAATGACGTGGGAGAAACA	120
S152	GACTACGACGATTTTGGGGTTC	GCAGATGTTGCTCTCGCTGT	114
S174	GGCTGGATGACTGGGTTTTATG	CCTGGGGTGTTTTGGGTATTT	100
S179	TTGATTACAAACGTGGGTGGTG	GCCCGTTGAGATAAGAGTTGGA	127
St15	CCGAGAAGAACGCTGATGG	AGCCGAGTTGGGACTTTTGT	83
St30	GGATTATTTTGGCATCACAGGAG	CAAGGGTCAAGTTTCGGTCAA	98
St37	TGCACCATTTATCTGAGCATCC	CCGCACACAACTCCATAACAA	93
St47	GAAGGGCGGTAGTTGTCCA	ACTCTTCCACCAGCATTTCCA	91
St53	TGTATGAACCAAAACCAGAGCA	AGGGGAATAGGAAGGGGAAG	115
St54	GCAGCCAACAAGAACA	AGTAGGCAACTTTAGC	124
St55	CCCAATACTAACCGCACAT	ACTCCTGATTACCCGACCC	333
St59	CATTGCCCAAGGTGTTCAT	CTGGTGTTTCGTCAGTAGC	101
St83	GCCCAACCAACATCACCA	TGTCGGTCGGAGCAAAGA	147
St95	GCACCACCCAGAATACCTT	GGGACCCAGCATACTCACG	299
St102	ACATCATCGTGACCTACTCGCTAC	CACACGACAGCATACACTCCAA	82
Actin	AGGCGGGATTTGCTGGT	TACCTCTTTTGGACTGGGCTTC	138

ethylene-responsive element binding protein, a putative auxin/aluminum responsive protein, an auxin-induced-related protein and jasmonate-*O*-methyl transferase (JMT). Real-time RT-PCR confirmed the induction of the auxin/aluminum responsive gene, whereas transcript levels for the other genes, due to the lack of specific primers, could not be determined. It is worth noting that an ethylene-responsive element binding factor (EREBF) has been suggested to regulate expression of ethylene-related genes, such as *acc2* in *A. thaliana* during *O. ramosa* infection (Vieira Dos Santos et al., 2003a). Furthermore, two genes involved in ethylene synthesis, *acco1* and *hacs1*, have been identified in sunflower during early infection by *O. cumana* (Letousey et al., 2007). Transcripts of related genes were up-regulated in *A. thaliana* in response to *O. ramosa* attack (Vieira Dos Santos et al., 2003b). Taken these data together, they suggest that ethylene signaling, and particularly EREBF proteins, may play an important role in *M. micrantha* plants parasitized by *C. campestris*.

Genes encoding auxin responsive proteins appear to have a function in defence responses against attack by fungal pathogens (Reddy et al., 2003), whereas transcripts encoding aluminum responsive proteins, known to play a role in plant responses to aluminum stress (Kumari et al., 2008), have been identified in *M. truncatula* upon *O. crenata* infection (Die et al., 2007). Increased transcript levels of the auxin/aluminum responsive gene in parasitized *M. micrantha* may reflect an adaptation of the host plant to *C. campestris* infection with respect to growth responses.

In the metabolic pathway of jasmonate, jasmonic acid carboxyl methyltransferase (JMT) is a key enzyme for the synthesis of methyl jasmonate, which acts as an important plant growth regulator mediating diverse developmental processes and

Table 5. Expression of *C. campestris*-induced *M. micrantha* genes measured by quantitative real-time RT-PCR.

Clone ID	Blast annotation	Induction fold
St15	Curculin-like lectin family protein	2.58
St30	Thiol-disulfide exchange intermediate	2.21
St37	Calcium dependent protein kinase 2	**5.35**
St47	Copper/zinc superoxide dismutase 1	**3.48**
St53	Thioredoxin family protein	**3.37**
St54	Glycine hydroxymethyltransferase	3.02
St55	Auxin signaling F-BOX 2	**3.51**
St59	S-adenosylmethionine synthase 3	**1.90**
St83	Fasciclin-like arabinogalactan protein 11	1.25
St95	Chitinase	1.49
St102	Tonoplast monosaccharide transporter 2	**7.25**
S18	Carbon-nitrogen hydrolase protein	2.00
S53	ATAF1 transcription factor	**5.23**
S56	Fatty acid dehydrogenase	1.30
S61	Auxin/aluminum responsive protein, putative	**3.07**
S83	NHL1(NDR1/HIN1-like1)	**1.95**
S91	Calcium exchanger 5	**5.21**
S120	Peroxidase, putative	1.09
S152	Glycolate oxidase	**6.18**
S174	NAP transcription factor	**5.02**
S179	Expansin A10	**3.27**

Notes: Values shown indicate the ratio of values from infected plants to non-infected control plants. Data indicate means from three independent replicates. Bold numbers indicate a statistically significant induction ($P \leqslant 0.05$).

defence responses. *JMT* responded to local and systemic signals generated by external stimuli, such as wounding and methyl jasmonate treatment (Seo et al., 2001). Moreover, other genes related to jasmonate signaling have been found to be up-regulated in host plants in response to *Orobanche* infection, such as *lox1*, *latex allergen*, *myrosinase binding protein* (*MBP*) and *lipoxygenase* (Letousey et al., 2007; Vieira Dos Santos et al., 2003a, b).

Genes involved in defence and stress reactions

In *M. micrantha* plants infected by *C. campestris*, expression of many genes known to be involved in defence and stress reactions seems to be induced. Genes belonging to this category include genes encoding copper/zinc superoxide dismutase, glycine hydroxymethyltransferase, chitinase, NHL1, glycolate oxidase and peroxidase. These findings are reminiscent to sequence data obtained from other parasitic plant–plant interactions. Although host responses to parasitic plants may vary in each interaction (Die et al., 2007; Letousey et al., 2007; Vieira Dos Santos et al., 2003b), it is likely that most host plants, at least to a certain extent, respond to parasitic plants with activation of plant defence responses.

Copper/zinc superoxide dismutases play an important role in cellular mechanisms against oxidative stress, which is often associated with other environmental stress factors. In leaves of cassava (*Manihot esculenta*) for example, transcript levels were strongly induced by high temperatures (37 °C), chilling (4 °C), the herbicide methyl viologen, and wounding (Shin et al., 2005). The remarkable increase of *copper/zinc superoxide dismutase* in shoots of parasitized *M. micrantha* suggests that protection from oxidative damage is a host response to *C. campestris* infection.

Chitinases are enzymes involved in plant defence against chitin-containing pathogens, such as fungi and arthropods. Expression of plant chitinase genes is often stimulated by biotic and abiotic stress factors (Collinge et al., 1993; Khan and Shih, 2004; Sahai and Manocha, 1993). A chitinase gene was also induced in sunflower during *O. cumana* infection (Letousey et al., 2007). The weak (non-significant) stimulation of *chitinase* expression in parasitized *M. micrantha* points to a non-specific reaction, as higher plants do not contain chitin.

Cell wall-related genes

Cell wall-related genes of *M. micrantha*, such as *β-galactosidase* and *polygalacturonase* were identified in this study by the SSH method. However, changes in their transcript levels during *C. campestris* infection were not confirmed by real-time RT-PCR, as we could not design appropriate primers. A β-galactosidase enzyme has been reported to cleave β-1,4 and β-1,3 linkages in galacto-oligosaccharides and to hydrolyze the pectic fraction of *A. thaliana* cell walls (Gantulga et al., 2008). Transcripts of polygalacturonase, a pectin-degrading enzyme, were found to be enhanced in tomato (*Lycopersicon esculentum*) during *C. reflexa* infection (Werner et al., 2001). In addition, a gene of *M. micrantha* encoding expansin A10 has been identified in this study. Expansin proteins are involved in cell expansion and cell wall-related developmental processes (Sampedro and Cosgrove, 2005). In *M. truncatula* infected by *O. crenata*, an expansin-like gene was remarkably up-regulated (Die et al., 2007). Based on these findings, we suggest that cell wall-modifying enzymes may

partially protect *M. micrantha* from *C. campestris* infection at early post-penetration stages.

Genes involved in transcriptional regulation

Transcription factors mediate regulation and expression of genes during diverse processes of plant development. Specific transcription factors are expressed in response to biotic and abiotic stress factors (Chen and Wang, 2002; Lin et al., 2007; Lu et al., 2007; Zhong et al., 2007). In this study, two NAC transcription factor genes (*ATAF1* and *NAP*) were found to be remarkably up-regulated in infected stems of *M. micrantha* (Table 5). Members of the NAC gene family contain a highly conserved NAC domain (for NAM, ATAF1/2, CUC2) and play an important role in diverse processes during senescence (Guo and Gan, 2006; Uauy et al., 2006), plant development (Zhong et al., 2007) as well as during biotic and abiotic stress conditions (Lin et al., 2007; Lu et al., 2007; Nakashima et al., 2007). In *A. thaliana*, ATAF1 negatively regulated the expression of stress responsive genes under drought stress conditions (Lu et al., 2007), whereas overexpression of *NAP* caused precocious leaf senescence (Guo and Gan, 2006). It is worth mentioning in this context that *C. campestris* absorbed water from its host (Dawson et al., 1994) and caused cell death in aerial parts of *M. micrantha* (Shen et al., 2005, 2007). It is tempting to speculate that ATAF1 in infected *M. micrantha* negatively regulates expression of stress responsive genes related to drought stress, and that NAP plays a role in leaf senescence in *M. micrantha* plants infected by *C. campestris*. Future work is required to elucidate the function of ATAF1 and NAP in the *M. micrantha*–*C. campestris* interaction.

Genes involved in transport processes

C. campestris absorbs water, carbohydrates and minerals from its host (Dawson et al., 1994) and the present study resulted in identification of genes of *M. micrantha* related to transport processes. The genes encoding tonoplast monosaccharide transporter 2 (TMT2) and calcium exchanger 5 (CAX5) showed significant increases in their transcript levels (Table 5). The TMT family comprises three isoforms in *A. thaliana*, which are involved in vacuolar sugar transport. *TMT1* and *TMT2* expression was up-regulated by stress factors, such as drought, salt, cold treatments and high sugar concentrations (Wormit et al., 2006). The function of *TMT2* during *C. campestris* infection remains unclear, but the 7-fold increase in transcripts suggests a crucial role in the *M. micrantha*–*C. campestris* interaction. CAX proteins are transporters that may modulate ion fluxes across the vacuolar membrane. Heterologous expression of *Arabidopsis CAX2* in tobacco increased the Cd^{2+} and Mn^{2+} transport in isolated root tonoplast vesicles (Hirschi et al., 2000).

Genes involved in signal transduction

Genes of *M. micrantha* involved in signal transduction during *C. campestris* infection were also identified in this study. Examples of this category are genes encoding a MAP kinase kinase protein, a kinase-related protein, a calcium-dependent protein kinase 2 (CDPK2), a calcium ion binding protein and a GTP-binding protein. The function of these genes in *M. micrantha* remains to be elucidated. The remarkable 5-fold increase in accumulated transcripts of *CDPK2* in infected *M. micrantha* plants indicates the involvement of stress-related signaling events in response to *C. campestris* infection. In fact, CDPKs play a crucial role in plant defence reactions under various biotic and abiotic stress conditions (Romeis et al., 2001; Tsai et al., 2007).

Finally, our work revealed an unexpected high number of *M. micrantha* ESTs encoding proteins with unknown function. It would be interesting to quantify the transcript levels of these genes in order to gain insights into the molecular function of these genes during *C. campestris* infection.

Taken together, this work represents a first attempt to identify genes preferentially or specifically expressed in shoots and stems of *M. micrantha* during *C. campestris* infection at early post-penetration stages. Sequence analysis of two SSH libraries resulted in 303 *M. micrantha* ESTs. Corresponding genes are predicted to encode proteins with various functions. Some of the identified ESTs were homologous to host genes up-regulated in other parasitic plant–plant interactions. Moreover, 16.29% of the *C. campestris*-induced ESTs in shoots and 17.60% of the *C. campestris*-induced ESTs in stems matched genes with unknown function in the NCBI database. Furthermore, real-time RT-PCR was used to estimate the expression of 21 representative *M. micrantha* genes and 13 of them showed significantly induction of expression levels in infected plants. Thus, sequencing of SSH libraries combined with real-time RT-PCR experiments has been successfully used to identify *M. micrantha* genes induced upon *C. campestris* infection.

The information generated in this study will be a good starting point for further research on the identified genes. Work in our laboratory is in progress to analyze the molecular function of these genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jplph.2009.02.002.

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