



Overexpression of stress-related genes in *Cuscuta campestris* in response to host defense reactions

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Abstract

Herb dodder (*Cuscuta* spp.) is one of the most important parasitic plants that can severely affect crop yields in the world. So far, interactions of this parasitic plant with hosts were not investigated adequately. Here, we conducted a differential expression analysis and identified a number of genes that were differentially expressed in haustorium tissue compared with the stem of *Cuscuta campestris* growing on Alfalfa. We obtained 439 cDNA fragments from haustoria (parasite-host connection zone) and stems (25 cm away from connections zones) using the cDNA-AFLP (Amplified Fragment Length Polymorphism) method with eight different primer combinations. Of 439 transcript-derived fragments (TDFs) that were detected, 145 fragments were identified as differentially expressed genes. Five TDF sequences were similar to known functional genes involved in signal transduction, metabolism, respiration, and stress responses. Genes encoding DEAD-box ATP-dependent RNA helicase, potential heme-binding protein, lysine-specific demethylase 5A were selected for qRT-PCR. The qRT-PCR analysis confirmed the results obtained using cDNA-AFLP. Our findings shed light on the elicitation of dodder defense responses in the connection zone to overcome plant defense reactions.

Key words: *Cuscuta campestris*, cDNA-AFLP, haustorium, Alfalfa

Introduction

The genus *Cuscuta*, commonly named dodder, includes 170 parasitic species that are distributed throughout the world (Holm et al., 1997). *Cuscuta* spp. thrive in areas with a warm and humid weather, where the maximum *Cuscuta*-dependent crop yield losses also occur (Dawson et al., 1994). However, *Cuscuta* spp. is found in all the regions of the world. Agriculturally, the most important *Cuscuta* spp. are *C. pentagona* and *C. campestris*, which show an almost global distribution and have a wide host spectrum. Severe crop damage due to *Cuscuta* is reported for 25 crop species in 55 kingdoms (Lanini and Kogan, 2005).

To this genus belong the holoparasitic flowering plants that must take up nutrients, water, and other macromolecules from the host plants with the help of specialized absorptive organs (Seel and Jeschke, 1999; Haupt et al., 2001; Birschwilks et al., 2006, 2007).

Cuscuta vines twine around the host stems or petioles and develop haustoria-root-like structures that penetrate the host tissues and fuse with the host vascular system to uptake water and nutrients. The process begins with the enlargement of *Cuscuta* epidermal cells and secretion of glue-like substances containing primarily de-esterified pectins that adhere to the host (Vaughn, 2002, 2003, 2006).

It was recently shown that *Cuscuta reflexa*'s attachment to tomato induces the host plant to synthesize arabinogalactan proteins (attAGP), which promote parasite observance (Albert et al., 2006). Following attachment, the haustorial cells elongate and penetrate the host tissues using both enzymes and mechanical pressure (Nagar et al., 1984). Individual cells of the haustoria elongate into search hyphae, which connect with the vascular bundles of the host (Birschwilks et al., 2006, 2007). On successful formation of vascular connections with the

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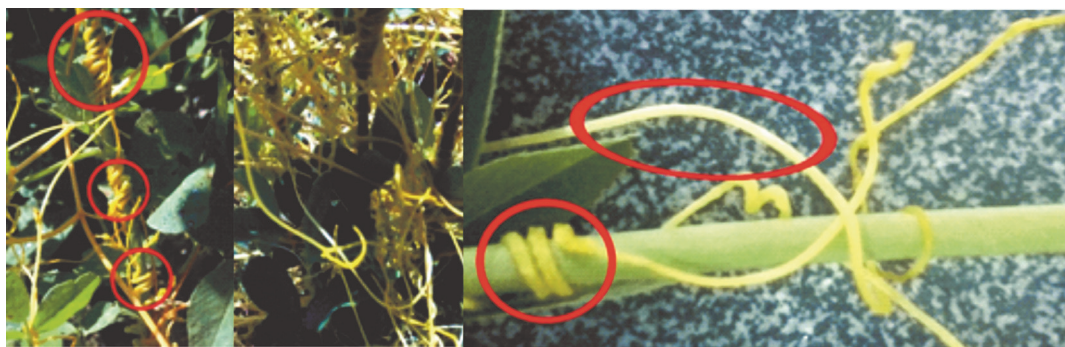


Fig. 1. Haustoria and stems of *Cuscuta campestris* coiled around Alfalfa stems; circles indicate regions selected for cDNA-AFLP analysis

host, *Cuscuta* becomes a powerful sink, uptaking water, sugars, amino acids, and other nutrients (Dawson et al., 1994; Birschwilks et al., 2007). *Cuscuta* are economically important pests as they damage numerous crops such as tomatoes and Alfalfa, as well as various species in natural habitats (Jiang et al., 2013).

On parasitization, dodder is intimately associated with the host plant. It can also benefit from herbicide resistance mechanisms of the host, making it difficult to attain an effective weed control without a detriment to the host (Cook et al., 2009; Nadler-Hassar et al., 2009). Agronomic and old breeding practices have additionally limited the ability to control the parasitic plant infection (Musselman et al., 2001; Mishra, 2009).

In farming zones, the control of dodder plants is very difficult due to the physiological resemblance and strong connections of the parasites with hosts. Therefore, increased understanding of the molecular mechanisms underlying the haustorium development and host attachment is vital to develop effective strategies for combating the negative effects of parasitic weeds (Aly, 2007; Alakonya et al., 2012; Jiang et al., 2013; Ichihashi et al., 2015). For the identification of the genes involved in the early stages of haustoria development, the suppression subtractive hybridization (SSH) technique was used; the genes involved in host–parasite interaction, haustorium initiation (pectin acetylesterase that plays a role in pectin modification during the infection process), oxidation of a number of substrates in young haustoria (genes encoding peroxidases), and also genes coding xyloglucan endotransglucosylase/hydrolase (XTH) that were highly and exclusively expressed at the onset of haustoria development, possibly involved in the invasive growth of the haustoria have been identified (Olsen et al., 2016).

Cuscutain (a cysteine protease from *Cuscuta reflexa*) playing a role in host–parasite interactions (Bleischwitz et al., 2010), genes encoding enzymes involved in cell wall modifications (Ranjan et al., 2014) taking part in haustorium development have been identified as well. Jiang et al., (2013) discovered ESTs (Expressed Sequence Tags) with sequence similarities to known plant viruses, including cryptic viruses, in the dodder sequence assemblies. They also determined that dodders are thought to have relatively robust antimicrobial defense capabilities. These data suggest that many genes of parasitic plants may be involved in attacks on the host plants and perform cell wall modifications in order to obtain nutrients and water. Our aim was to identify the genes involved in defense in dodder in its connection site with the Alfalfa host during the attachment phase.

Materials and methods

Plant material

Cuscuta campestris were propagated on a compatible host Alfalfa (Fig. 1) in a field in the campus of Ramin Agriculture and Natural University (Khozestan-Iran).

Inoculation with C. campestris and collection of samples

To create a connection zone in the host–parasite systems, about 30 cm long *Cuscuta campestris* stems were coiled around stems of 2.5-month-old Alfalfa plants. Noninfective regions of dodder stems (25 cm away from host plants) and haustoria were collected separately 3 days post infection, because at this time the first visible haustoria were established. The collected samples were washed with distilled water, dried with filter paper, and then immediately frozen in liquid nitrogen and stored at -80°C .

Table 1. Primer sequences, designed from sequenced transcript-derived fragments, used for qRT-PCR validation analyzes

Primer ID	Size (nt)	T_a [°C]	Forward primer	Reverse primer
Actin	100	57	ATGGAAGCTGCTGGAATCCAC	TTGCTCATACGGTCAGCGATG
ATP	117	59	CCTGGCTGTTGGGATTTC	CATTGGCAAGGTGGATGAG
Lys	100	59	ATCACATAGAAGCATCACCTC	TGAGAGACATGATGAAGTGAAC

T_a – annealing temperature

RNA extraction and cDNA synthesis

Total RNA was isolated from 100 to 200 mg of frozen tissue material (stem and haustorium), using RNeasy Mini Kit (Qiagen, USA) according to manufacturer's instructions. Quality and quantity of isolates were analyzed on 1% (w/v) agarose gels in 1x TBE buffer, using a NanoDrop spectrophotometer. Single-stranded cDNA (ss-cDNA) was synthesized with Prime Script First Strand cDNA Synthesis Kit protocol (cDNA Synthesis Kits, Takara, Japan). Double-stranded cDNA (ds-cDNA) was synthesized using 20- μ l first strand cDNA, RNase H (5 units/ μ l), DNA Polymerase I (10 units/ μ l), T4 DNA Polymerase (Thermo Scientific, USA), and 10 mM of dNTP mix, at 15°C for 2 h. The resulting ds-cDNA was purified using 1 volume of phenol: chloroform: isoamyl alcohol (25:24:1) plac. The pellets were washed, dried, and dissolved in 40 μ l of nuclease-free water. Five microliters from ds-cDNA of each sample was analyzed on 2% (w/v) agarose gel in 1 \times TBE buffer.

cDNA-AFLP analysis

About 600 ng of ds-cDNA were used for the cDNA-AFLP analysis. Ds-cDNA was digested for 3 h at 37°C with *Eco*RI, and 2 h at 65°C with *Tru*I1 (*Mse*I) (Thermo Scientific, USA). Then, the reaction mixtures were incubated for 15 min at 80°C to inactivate the enzymes. The digested products were ligated to *Eco*RI and *Tru*I1 dsDNA adapters by T4 DNA Ligase (Thermo Scientific, USA) for 2 h at 22°C and then incubated for 15 min at 65°C. The preselective amplification mixture was prepared using 1/3 volume of the digested and ligated cDNA (as template) and primers corresponding to *Eco*RI and *Tru*I1 adapters with an additional selective base (*Eco*RI, 5'-GACTGCGTACCAATTCA-3' and *Mse*I, 5'-GATGAGTCCTGAGTAAC-3') for 23 cycles (94°C, 30 s for denaturation; 56°C, 60 s for annealing;

and 72°C, 60 s for extension). The PCR product was diluted (30-fold) with nuclease-free water. Then, 2 μ l were used for PCR amplification using 8 combinations of selective *Eco*RI and *Tru*I1 primers with three additional nucleotides at their 3'-ends (4 primers for *Tru*I1+3 (AAG and ACC) and 2 primers for *Eco*RI+3 (CAA, CAC, CAT, CAG) (Akash et al., 2013). Next, PCR was performed as follows: denaturation 5 min 94°C, 13 cycles of 94°C for 30 s, 65°C for 30 s (–0.7°C, in each cycle) and 72°C for 60 s; then, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s, and 72°C for 10 min. To each PCR product, 5 μ l of DNA sequencing stop solution was added, and 9 μ l of each sample was separated on a 6% (w/v) polyacrylamide gel run at 1800 V and 50 W and 45°C for 2 h (Bachem et al., 1996). Amplicons were visualized by silver staining (Bassam et al., 1991).

Bands with differences in intensities, and polymorphic transcript-derived fragments (TDFs, chosen based on a presence or absence of bands in haustorium compared with the stem) were excised from the gel with a scalpel, and placed in 50 μ l of nuclease-free water at 4°C overnight and then stored at –20°C. About 3 μ l of each sample was used for reamplification in 20 μ l using the same set of selective primers and PCR program as used for the preamplification (annealing temperature of 56°C for 35 cycles). PCR products were resolved in a 2% (w/v) agarose gel in 1 \times TBE buffer; each transcript was isolated and purified using the DNA gel extraction kit (Vivantis).

Sequencing of TDFs

Among the 145 fragments, 20 TDFs that differed between the haustorium and the stem from the haustorium area (connection zone with host) were sequenced at Macrogen-Korea. TDFs sequences were analyzed using BLASTx and BLASTn programs of the National

Center for Biotechnology Information database (NCBI: <http://www.ncbi.nlm.nih.gov>).

Real-time RT-PCR analysis

Real-time RT-PCR analysis was performed to verify the expression levels of three selected sequences (numbered 2, 4, and 6; details are given in Table 1) with known functions in haustorium. Single-stranded cDNA was synthesized from 1 µg of the total RNA (treated with RNase-free DNase) using the First-Strand cDNA Synthesis Kit (TaKaRa, Japan) following the manufacturer's instructions. Forward and reverse real-time PCR primers specific for chosen TDFs were designed using Integrated DNA Technologies database (IDT: <https://eu.idtdna.com/Primerquest/Home/Index>) (Table 1). Quantitative RT-PCR (qRT-PCR) was performed using Step One Plus Real-Time PCR System by HiFi SYBR Mix Plus (Takara, Japan). The total volume of reactions were 25 µl, including 1 µl (0.2 µM) of each primer, 2 µl HiFi SYBR Mix Plus, 2 µl of diluted (1/10) first strand cDNA, and 4 µl nuclease-free water. PCR conditions were as follows: denaturation for 10 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for 20 s at 72 °C, and one more elongation step at the end for 15 s at 72 °C. For the determination of relative quantification of gene expression a housekeeping (*Cr*)-*ACTIN* gene was used as a reference.

Results

The stem and haustorium of dodder (connection and nonconnection regions to a host plant) were used for cDNA-AFLP transcript profiling to identify genes that were differentially regulated during the infection process.

The analyzed eight primer combinations allowed for the visualization of 439 TDFs. Per primer combination, on average, 55 TDFs were visualized as bands, from 30 to 600 bp in size. To determine the reproducibility of these band profiles, the experiments were repeated twice (Fig. 2).

Among 439 bands obtained in the analysis, 145 bands showed significantly different expression patterns (based on their presence/absence or difference in the levels of expression) between *C. campestris* stem and haustorium. About 9.06 % and 31.9% of these fragments were up- or downregulated, or present/absent, respectively (Table 2 and Fig. 3 and Fig. 4).

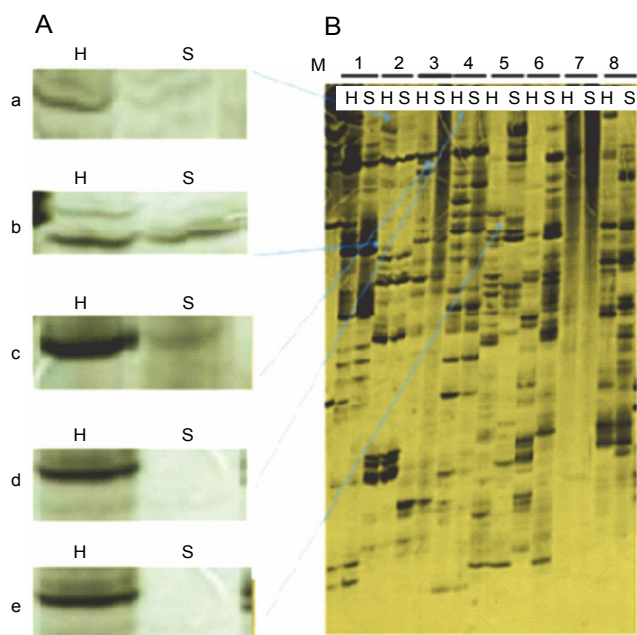


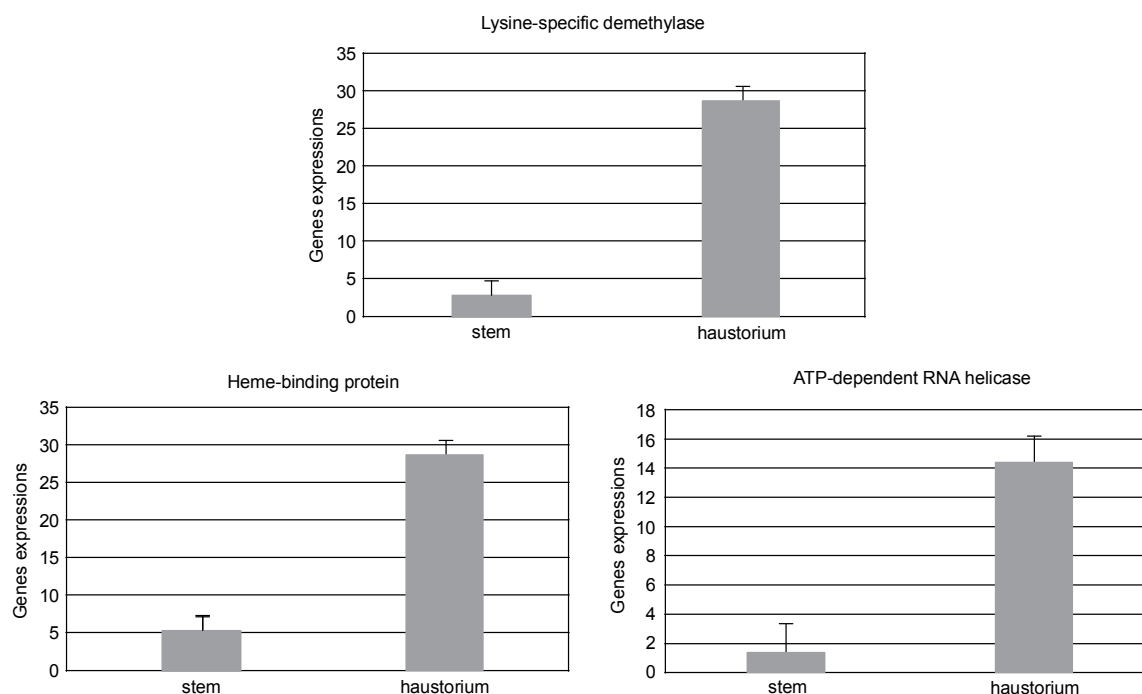
Fig. 2. A – magnification shows different expression patterns in the stem compared with that in the haustorium and some products separated and sequenced; In parts a, b, d, and e bands were present in the haustorium and absent in the stem of *C. campestris*, but in part c, TDF was upregulated in the haustorium and downregulated in the stem; B – silver-stained cDNA-AFLP gel showing differential expression of TDFs obtained using 8 primer combinations; numbers: 1 – *EcoRI* AAG-*TruI* CAA, 2 – *EcoRI* AAG-*TruI* CAT, 3 – *EcoRI* AAG-*TruI* CAG, 4 – *EcoRI* AAG-*TruI* CAC, 5 – *EcoRI* ACC-*TruI* CAA, 6 – *EcoRI* ACC-*TruI* CAT, 7 – *EcoRI* ACC-*TruI* CAG, and 8 – *EcoRI* ACC-*TruI* CAC; the samples from left to right in each combination are haustorium (H) and stem (S)

Sequence search of these TDFs against NCBI databases was done for identification and to determine their functional classes. Sequence annotation was performed from homology search obtained using BLASTx and BLASTn programs in NCBI database (Table 3) and TDFs were classified in different functional groups. The sequenced genes were assigned to signal transduction, metabolism, respiration, and stress response groups. However, TDF8, TDF9, and TDF10 had no similar sequences in any of the databases searched. These sequences may thus represent genes that have not yet been identified and that are highly expressed in the haustorium of *C. campestris*.

To verify the data obtained from cDNA-AFLP analysis, the expression of three genes (potential heme-binding protein, lysine-specific demethylase 5A, ATP-dependent RNA helicase) were monitored by relative-quantitative RT-PCR. In all the cases, the expression profiles

Table 2. Present, absent, and up- or downregulated TDFs detected in the stem and the haustorium of *C. campestris* obtained using eight primer combinations

Primer mix	Tissue	Number of TDFs	Different polymorphism	Upregulated	Polymorphism (%)	
					up- or downregulated	presence or absence
AAG/CAA	stem	18	2	0	0	11.11
	haustorium	27	16	0	0	59.26
AAG/CAT	stem	27	13	0	0	48.14
	haustorium	11	3	0	0	27.27
AAG/CAG	stem	21	15	0	0	71.42
	haustorium	19	10	0	0	52.36
AAG/CAC	stem	32	21	0	0	65.62
	haustorium	20	5	0	0	25
ACC/CAA	stem	34	1	2	5.9	2.94
	haustorium	48	12	5	10.41	25
ACC/CAT	stem	37	4	2	5.4	10.81
	haustorium	36	5	10	27.8	13.88
ACC/CAG	stem	12	0	0	13.04	0
	haustorium	41	28	6	13.04	68.29
ACC/CAC	stem	23	0	0	0	0
	haustorium	33	10	0	0	30.30
Total		439	145	27	9.06	31.90

**Fig. 3.** Quantitative RT-PCRs validate the expression of TDFs in cDNA-AFLP analysis; expression patterns of heme-binding protein; expression patterns of lysine-specific demethylase; expression patterns of ATP-dependent RNA helicase

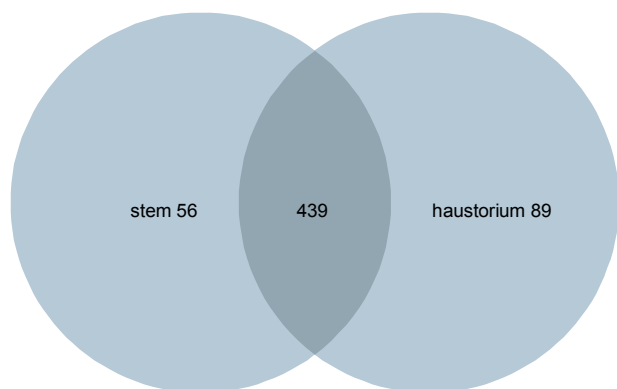


Fig. 4. Numbers of genes showing significantly different expression patterns between the stem and a haustorium of *C. campestris*

were consistent with the results obtained from the cDNA-AFLP analysis (Fig. 3).

High expression levels of TDFs no. 2, 4, and 6 in the haustorium compared with the dodder stems (11.48-, 11-, and 5.2-fold upregulated, respectively), indicate expression pattern correctly under the attachment conditions.

Discussion

Development of effective strategies for controlling the spread of dodder in the fields can be available only through learning more about different aspects of interactions between *Cuscuta* and its hosts and also about the aspects of molecular mechanisms of parasite invasion. Our findings provide new evidence supporting important roles of some genes during dodder invasion. Those genes have also been implicated in stress responses. Comparative transcriptomic analysis such as cDNA-AFLP provided a valuable resource for studying host–dodder interactions in the past (Ranjan et al., 2014; Breyne and Zabeau, 2001). In our research, the highest upregulation in cDNA-AFLP analysis was observed for genes encoding for heme-binding protein, lysine-specific demethylase, and ATP-dependent RNA helicase. These data were confirmed by real-time PCR showing the overexpression of these genes in haustorium compared with the stem tissue of *Cuscuta*. Our data support the thesis that these genes could be involved in facilitating haustorium invasion on a host plant.

The sequence of TDF2 (upregulated in haustorium tissue compared with dodder stems) was annotated in NCBI database as DEAD-box ATP-dependent RNA helicase. DEAD-box ATP-dependent RNA helicases belong

to the largest family of RNA helicases. They use energy derived from ATP hydrolysis to promote the proper folding of RNA molecules (Tanner and Linder, 2001). Evidence suggests that this candidate gene regulating RNA metabolism (Zhu et al., 2015) is also involved in a transfer of mRNAs (Itadani et al., 1994) and microRNA biogenesis (Park et al., 2002; Reinhart et al., 2002). Based on these findings, we have proposed that DEAD-box ATP-dependent RNA helicases may regulate the movement of RNAi signals through parasite bridges from host tissues into *Cuscuta*. Exchange of mRNAs between a parasitic plant and its host was reported earlier (Kim et al., 2014). Therefore, the overexpression of DEAD-box RNA helicase in haustorium can be explained by the crucial role played in the transfer of mRNA or RNAi signals between the parasite and its host and also important roles played in regulating different defense signaling pathways against biotic and abiotic stresses (Li et al., 2008). Several DEAD-box RNA helicase transcripts were identified through genome-wide expression profiling analyzes, which suggest these genes might be differentially regulated under stress conditions such as cold and chilling (Seki et al., 2002; Kreps et al., 2002; Gong et al., 2005; Kant et al., 2007). The DEAD-box RNA helicase's performance during stress conditions have not yet been fully specified or understood as a result of poor knowledge on the enzymes in plants.

TDF4 sequence, which corresponds to the potential heme-binding protein, is upregulated in haustorium. The presence of a large number of heme carrier proteins supports the hypothesis that heme is more suitable as a drought stress signal stimulating ROS detoxification under drought stress (Nagahatenna et al., 2015). It has been proposed that heme could posttranslationally activate specific transcription factors that are necessary for stress acclimation (Nagahatenna et al., 2015). Defense mechanisms may be induced by dodder parasitism in the host plants (Runyon et al., 2010). Interestingly, the parasitic plant is also under biotic stress and uses its own defense mechanisms to overcome the host's counter attack (Ranjan et al., 2014). Some stress conditions modulate heme synthesis (DeFranceschi et al., 2011); similarly to other heme-binding proteins, it may be involved in the cellular protection against heme-mediated oxidative damage to the cell (Immenschuh et al., 1999).

Nucleotide sequence of TDF6 is similar to lysine-specific demethylase 5A. Based on the real-time PCR

Table 3. TDFs differentially expressed in the haustorium that are similar to genes encoding proteins with known functions

TDF _s	Prime name	Size (bp)	Annotation	ID	Species	E-values
Nucleic acid metabolism 2	E-ACC / M-CAT	302	DEAD-box ATP-dependent RNA helicase	80020935	<i>Vitis vinifera</i>	2e-60
Signal transduction 3	E-AAG / M-CAG	129	calcium-dependent protein kinase (CPK5) mRNA	80020936	<i>Nicotiana tabacum</i>	5e-19
Photosynthesis and energy 4	E-ACC / M-CAC	79	potential heme-binding protein	80020937	<i>Cuscuta gronovii</i>	4e-23
Cell defense and stress 5	E-AAG / M-CAA	167	GPI mannosyltransferase 1	80020938	<i>Nicotiana sylvestris</i>	3e-18
6	E-AAG / M-CAA	219	lysine-specific demethylase 5A	80020939	<i>Beta vulgaris</i>	9e-26
8	E-AAG / M-CAT	169	no significant similarity	80020940		
9	E-AAG / M-CAT	167	no significant similarity	80020942		
10	E-AAG / M-CAG	319	no significant similarity	80020943		

analysis, the expression level of lysine-specific demethylase gene increased by 11-fold in the haustorium tissue compared with that in the stem. Methylation of lysine residues within histone tails is an important modification that contributes to an increase in gene expression levels or their repression depending on the specific residue methylated and the degree of methylation (Heightman, 2011). Therefore, lysine-specific demethylase 5A plays an essential role in regulating various developmental processes (Liu et al., 2010), transcriptional regulation (Zhou et al., 2010), and plant responses to various stresses (Yuan et al., 2013).

TDF3, which corresponds to calcium-dependent protein kinase, is upregulated in haustorium. Calcium levels, as molecular signaling molecules, are increased by a diverse physiological stimuli and stresses. Calcium-dependent protein kinases are plant proteins that directly bind calcium ions and therefore play important roles as Ca²⁺ sensor proteins in transducing differential Ca²⁺ signatures, that are triggered by effectors and activating complex downstream responses (Gao et al., 2014). They are involved in metabolism, osmosis, hormone response, and stress signaling pathways (Valmonte et al., 2014).

The sequence of TDF2 was annotated as glycosylphosphatidylinositol mannosyltransferase 1 (GPI-MTs)

that is essential for glycosylphosphatidylinositol (GPI) biosynthesis, that GPI-anchored proteins serve essential functions as cell adhesion molecules (CAMs), hydrolytic enzymes, and are also attached to cell-wall glycoproteins compounds, for example attached to Arabinogalactan proteins (AGPs) that belong to the hydroxyproline-rich superfamily of glycoproteins and are known to be involved in many biological processes like cell division, cell elongation, and cell death (Spree et al., 1994; Gao et al., 1999; Van Hengel et al., 2002; Gillmor et al., 2005). Cell walls play an important role in plant protection against stress conditions (Striberny and Krause, 2015). Various observations support the hypothesis that through connections formed between dodder and the host plant, transfer of nutrients, water, and even proteins, viruses, or RNAs into the parasitic plants or in a bidirectional manner occurs (Haupt et al., 2001; Hibberd et al., 1998; Albert et al., 2006; Fürst et al., 2016).

Conclusions

Based on our findings, we hypothesize that defense responses were elicited in dodder to overcome host defense responses in connection zones. *Cuscuta* parasites may encounter miRNA signaling molecules that are

generated in stressed plants (Stefanovic et al., 2002). We observed that the migration of signals from the host to the parasitic plant induced the expression of specific genes to overcome plant defense responses. In our study, several genes with different functions were identified, such as ATP-dependent RNA helicase, glycosylphosphatidylinositol mannosyltransferase 1 (GPI-MTs), calcium-dependent protein kinase, and heme-binding protein. These findings could help further knowledge on the interaction between the host and the parasite.

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