A survey of genes involved in *Arachis stenosperma* resistance to *Meloidogyne arenaria* race 1

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Abstract. Root-knot nematodes constitute a constraint for important crops, including peanut (*Arachis hypogaea* L.). Resistance to *Meloidogyne arenaria* has been identified in the peanut wild relative *Arachis stenosperma* Krapov. & W. C. Greg., in which the induction of feeding sites by the nematode was inhibited by an early hypersensitive response (HR). Here, the transcription expression profiles of 19 genes selected from *Arachis* species were analysed using quantitative reverse transcription–polymerase chain reaction (qRT-PCR), during the early phases of an *A. stenosperma–M. arenaria* interaction. Sixteen genes were significantly differentially expressed in infected and non-infected roots, in at least one of the time points analysed: 3, 6, and 9 days after inoculation. These genes are involved in the HR and production of secondary metabolites related to pathogen defence. Seven genes encoding a resistance protein MG13, a helix-loop helix protein, an ubiquitin protein ligase, a patatin-like protein, a catalase, a DUF538 protein, and a resveratrol synthase, were differentially expressed in all time points analysed. Transcripts of two genes had their spatial and temporal distributions analysed by *in situ* hybridisation that validated qRT-PCR data. The identification of candidate resistance genes involved in wild peanut resistance to *Meloidogyne* can provide additional resources for peanut breeding and transgenic approaches.

Additional keywords: hypersensitive response, *in situ* hybridisation, peanut, qRT-PCR, root-knot nematode, wild *Arachis*.

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are the most frequently observed biotrophic nematode parasites in plants, with a broad host range of more than 2000 species, including many crops (Agrios 2005). These nematodes are obligate endoparasites that occur in tropical and temperate regions, and cause significant economic losses in peanut production areas worldwide (Holbrook *et al.* 2000). The most widely used strategy to control root-knot nematodes is the use of nematicides, which are often highly toxic and have been progressively restricted or banned due to environmental and human health concerns (Collange *et al.* 2011). The use of resistant cultivars is one of the most promising practices to reduce crop damage in an integrated pest management approach. Indeed, host resistance against nematodes has been identified in many crop species, including

soybean (Davis *et al.* 1998), cowpea (Olowe 2009), coffee (Albuquerque *et al.* 2010), and also peanut, *Arachis hypogaea* L. (Castillo *et al.* 1973). Resistance against *M. arenaria* race 1, the most damaging nematode for peanut, has been observed in the wild species *Arachis cardenasii* Krapov. & W. C. Greg., *Arachis batizocoi* Krapov. & W. C. Greg. and *A. stenosperma* Krapov. & W. C. Greg., as shown by the decrease in the number of females and the delay in the pathogen life cycle (Nelson *et al.* 1990; Choi *et al.* 1999; Bendezu and Starr 2003). Further, two peanut cultivars, COAN and Nematan, resistant to this nematode were developed by introgressing a dominant gene from *A. cardenasii* (Nagy *et al.* 2010). It is important that new sources of *Arachis* resistance to *M. arenaria* are identified in *Arachis*, in order to decrease the risk of resistance breakdown due to the emergence of new virulent nematode populations.

Nematode infection initiates complex changes in plant gene expression (Williamson and Gleason 2003; Davis and Mitchum 2005), starting with infective second-stage nematode juveniles (J_2) entering host roots and migrating toward the vascular cylinder in search of suitable cells for initial feeding sites establishment. J_2 then initiate localised reorganisation of host cell morphology and physiology, resulting in the formation of specialised feeding cells (Jones 1981). In the case of *Meloidogyne* spp., the feeding cells enlarge and become multinucleated through divisions in absence of cytokinesis, and are called 'giant cells' (Huang 1985). The understanding of cell and molecular mechanisms of host–pathogen interactions may provide additional resources for developing new forms of durable plant defences (Williamson and Kumar 2006).

A genome overview of gene expression in *Arabidopsis* infected with *M. incognita* by microarray analysis showed that approximately 15% of the genes displayed significant differential expression between non-infected roots and nematode galls at different stages of the infection, evidence of the complexity of nematode feeding-site development (Jammes *et al.* 2005). Likewise, altered expression of numerous genes during the interaction with *Meloidogyne* spp. in soybean roots has also been demonstrated (Ibrahim *et al.* 2011; de Sá *et al.* 2012).

So far, the mechanisms of resistance of wild *Arachis* to root-knot nematodes have been characterised in detail only in *A. stenosperma*, which shows typical symptoms of an early hypersensitive response (HR). In this species, the induction of nematode feeding sites was associated with a necrotic-like response, thereby hindering nematode development, root-galling and egg mass formation (Proite *et al.* 2008).

Nematode infection triggers pathways related to defence response, including HR genes, as well as those involved in the redesign of root morphology to form galls and giant cells for feeding (Ibrahim *et al.* 2011). As a vast suite of genes are induced in defence response against root-knot nematodes and other pests, a specific recognition of the pathogen by the plant may lead to resistance through HR, accompanied by rapid cell death in and around the infection site, thus, effectively containing pathogens at their site of entry (Lam 2004). Early HR against different species of *Meloidogyne* has been described in several plants, such as cotton (Mota *et al.* 2012), wild peanut (Proite *et al.* 2008), *Mi-1*-mediated resistance in tomato (Vos *et al.* 1998), *Mex-1*-mediated resistance in coffee (Anthony *et al.* 2005), and *Me3*-mediated resistance in pepper (Pegard *et al.* 2005). In addition, complex networks of genes that interfere in metabolic pathways, cell-cycle progression and water transport are among those whose expression is increased in the feeding cells as well as in the resistance response (Williamson and Gleason 2003), which demonstrates the enormous challenge to select and identify reliable candidate genes for further validation and application to broadening plant resistance to root-knot nematode.

In peanut and its wild relatives, only a few studies analysing transcriptome profiles have dealt with plant–nematode interactions. In cultivated peanut infected with *M. arenaria*, Tirumalaraju *et al.* (2011) showed several genes were differentially expressed in resistant and susceptible cultivars. Likewise, the analysis of expressed sequence tags (ESTs) derived from wild *A. stenosperma* revealed a number of

differentially expressed genes between inoculated and control plants; these being related to hormonal balance, defence and alleviation of oxidative stress triggered by HR (Proite *et al.* 2007; Guimarães *et al.* 2010).

In the present study, the expression profiles of 19 nematode resistance candidate genes selected either from our wild *Arachis* EST databanks or public databases were analysed using quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and *in situ* hybridisation. Analyses focussed on the early phases of *A. stenosperma–M. arenaria* interaction, with the aim to identify those genes potentially involved in triggering the HR response and inhibition of giant cell formation.

Materials and methods

Plant material and nematode challenge

Seeds of *Arachis stenosperma* Krapov. & W. C. Greg. (accession V10309), resistant to *Meloidogyne arenaria* race 1, and *A. hypogaea* L. cv. Florunner, a susceptible cultivar, were treated with Ethrel (Bayer Crop Science, Research Triangle Park, NC, USA) 0.1% (v/v) for 12 h, and planted singly in 600 cm^3 pots containing steam-sterilised soil. Plants were maintained under greenhouse conditions at the University of California, Riverside, USA, at $28-35°C$ air temperature, and watered daily. Eggs of *M. arenaria* race 1 were cultured on susceptible tomato cv. UC82 host plants, extracted from roots using 10% (v/v) bleach solution (Hussey and Barker 1973) and hatched at 28°C in an incubator. Approximately 20 000 juveniles (J_2) resuspended in fresh deionised water were pipetted onto soil depressions around each of the 4-week-old plants of *A. stenosperma* and *A. hypogaea*. Control individuals received mock inoculum consisting of deionised water only. Plants were arranged randomly on a bench, and roots collected at 0, 3, 6, and 9 days after inoculation (DAI). Collected roots were either immediately frozen in liquid nitrogen and stored at $-80°C$ for RNA extraction or immersed in a cacodylate solution containing glutaraldehyde and paraformaldehyde for *in situ* hybridisation procedures, as detailed below.

RNA extraction and cDNA synthesis

Total RNA was extracted from roots (250 mg) of *A. stenosperma* individual plants, following the lithium chloride modified protocol described by Morgante *et al.* (2011), and purified with Invisorb Plant RNA Mini Kit (Invitek, Berlin, Germany). Total RNA integrity was checked by 1% (v/v) gel electrophoresis and its concentration was quantified on NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Equal amounts of total RNA per collecting point (0, 3, 6, and 9 DAI) were pooled from three plants, in two independent biological replicates. A total of 2 μg of each RNA pool was treated with 2 U of DNase (Fermentas, St Leon-Rot, Germany) to eliminate genomic DNA, and reverse transcribed using the Super Script II enzyme and oligo(dT)$_{20}$ primer (Invitrogen, Carlsbad, CA, USA), according to manufacturer's instructions. DNAse treatment and cDNA synthesis were performed in subsequent steps, in the same tube, to avoid nucleic acid loss during precipitation and washing steps. cDNA samples were diluted (1 : 100) and stored at $-20°C$.

Elimination of DNA contamination in cDNA samples was further checked by real-time PCR using primers from *Arachis magna* Krapov. & W. C. Greg. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) coding gene (GAPDH-F 5′-CAA CAACGGAGACATCAACG-3′ and GAPDH-R 5′-ATCACT GCCACCCAGAAAAC-3′; Morgante *et al.* 2011) that flank an intron region in *Arachis* spp.

Candidate gene selection

Nineteen candidate genes were selected for transcriptional profile analysis in plants of *A. stenosperma* challenged with nematodes by using qRT-PCR (Table 1). Twelve genes were chosen based on our previous work (Proite *et al.* 2008; Guimarães *et al.* 2010) and our wild *Arachis* transcripts database. The remaining genes were selected considering their role in root-knot resistance in plants (Jammes *et al.* 2005; Tirumalaraju *et al.* 2011; Kyndt *et al.* 2012) and *A. stenosperma* homologous sequences identified using BLAST tools (Altschul *et al.* 1990).

For each of the 19 candidate genes, primers were designed based on the contig alignments, using Primer 3 Plus software (Untergasser *et al.* 2007), with lengths between 19 and 22 nucleotides, melting temperature between 55 and 62°C, G/C content between 45 and 55%, and PCR products ranging from 100 to 210 bp. Gene-specificity of primers was confirmed using BLAST searches on the *A. stenosperma* transcripts database resources (Expressed Sequence Tags (EST) and Transcriptome Shotgun Assembly (TSA)) from the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/, accessed 10 May 2012). Amplicon length was checked by real-time PCR using an equimolar pool of all cDNA samples as template. Primer sequences, length of products, and source of sequences are listed in Table 1.

qRT-PCR analyses

qRT-PCR assays were performed using 5 µL of Platinum SYBR Green qPCR Super Mix-UDG w/ROX kit (Invitrogen) in a 10 µL final volume reaction containing 2 µL of diluted cDNA and 0.2 µM of each primer. GAPDH and ribosomal 60S coding genes were used as reference for data normalisation (Morgante *et al.* 2011). Reactions were conducted in three technical replicates for each sample in 96-well plates on the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The program applied was: 2 min at 50°C; 10 min at 95°C, followed by 40 cycles of 95°C (15 s) and 60°C (1 min). Dissociation curve analysis of amplification products was performed for each reaction.

Primer efficiency and optimal cycle of quantification (Cq) values were estimated using the online real-time PCR Miner tool (Zhao and Fernald 2005). Average Cq values were normalised to the two reference genes (*AsGAPDH* and *As60S*), and expression ratios of mRNA transcripts at 3, 6, and 9 DAI, relative to day 0, were calculated and statistically tested using REST 2009 ver. 2.0.13 software (Pfaffl *et al.* 2002). Gene expression values were further represented in a heat map using GENE-E tool (http://www.broadinstitute.org/cancer/software/GENE-E/, accessed 13 January 2013).

In situ *hybridisation*

Fixed roots were gradually dehydrated in a graded series of ethanol solutions (70, 90, 96, and 100%) and slowly embedded in butyl-methyl methacrylate. Semi-thin sections with 2.5–3.5 µm of thickness were mounted on histological slides and resin removed from the sections by repeated washes with acetone. One slide of each material was stained with acridine orange in order to confirm RNA preservation of processed samples.

The mRNA sequences of *A. stenosperma* genes coding an auxin-repressed protein (*AsARP*) and a cytokinin dehydrogenase 3-like (*AsCKX*) were amplified and cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). The plasmids were linearised by digestion with *Sac* I or *Sac* II restriction enzymes and used as probes, after labelling with the Digoxigenin RNA *in vitro* transcription labelling kit (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions. Root sections were pre-treated and hybridised with 2 ng of the labelled probe in 1 µL of hybridisation solution in a humid chamber at 42°C for 12 h, as described by Tucker *et al.* (2003). Hybridisation sites were immunocytochemically detected using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche), followed by incubation with NBT/BCIP buffer (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) for colorimetric detection of the phosphatase activity. Slides were mounted in glycerol and analysed under an epifluorescent microscope (Carl Zeiss, Zeiss, Germany).

Results

In order to identify and characterise genes involved in the incompatible interaction between *A. stenosperma* and *M. arenaria* race 1, the transcriptional profile of 19 candidate genes potentially involved in the resistance response were analysed by qRT-PCR, and, in some cases, also by *in situ* hybridisation, during the early stages of the infection.

Nematode infection and RNA extraction

Roots from the resistant *A. stenosperma* were inoculated with J$_2$ stage of *M. arenaria* race 1 and the onset of disease symptoms was compared with non-infected roots. Susceptible *A. hypogaea* cv. Florunner plants, used as positive controls for the nematode infection, showed swollen roots corresponding to nematode feeding sites around 9 DAI, and numerous root-galls at 30 DAI (data not shown). As expected, no infection symptoms were visible in the root systems of inoculated *A. stenosperma*. This is in line with our previous work where no tissue hypertrophy or cell hyperplasia was observed after nematode inoculation in the resistant species *A. stenosperma* (Proite *et al.* 2008).

Total purified RNA from all root samples showed high quality and the formed pools were reverse transcribed for qRT-PCR analysis. The absence of genomic DNA was confirmed by using *GAPDH* primers, as the expected amplicons have 190 and 340 bp from cDNA and genomic templates, respectively, allowing the distinction between PCR products. The different sizes are apparently due to the presence of an intron flanked by *GAPDH* primers. These primers, originally designed for *A. magna* (Morgante at al. 2011), also generated distinct

Table 1. Genes and primers used for qRT-PCR analysis

GenBank accession	Putative gene BlastX first hit	Related taxon	E-value	Amplicon size (bp)	Amplification efficiency ± s.d.	Primer sequences Forward	Reverse
EH048133	Resveratrol synthase (RS)	*Arachis hypogaea*	8e-116	101	1.04 ± 0.006	5′-CAAGGATGCTCGTGTGCTTA-3′	5′-GGCACAGGATCAGAACCAAT-3′
EH046566	Hypothetical protein (HPNt)	*Nicotiana tabacum*	3e-48	119	0.99 ± 0.008	5′-GAGAAGGAAGGACGCTTTCA-3′	5′-AAAGTCCAAAGAGCGCAGAC-3′
EH045758	DUF538 (DUF)	*Glycine max*	5e-111	154	1.05 ± 0.02	5′-GAGCACAGGGTACATGTGGA-3′	5′-CATAAGGTCCTTGGCCTTCA-3′
EH044463	Calmodulin (CaM)	*Ricinus communis*	1e-90	158	0.99 ± 0.007	5′-ACAAGGACAACACCGGCTAC-3′	5′-CATTCTGGCGATGAAATCCT-3′
EH046375	Nuclear factor Y (NF-Y)	*Medicago truncatula*	3e-116	174	0.97 ± 0.007	5′-GCTAGCATCGATCCCAAGAG-3′	5′-GGAGCCATGTAGGGATGAGA-3′
EH047440	Resistance protein MG13 (MG13)	*Glycine max*	1e-54	190	1.04 ± 0.006	5′-AATTGAAGCGTCCAGGATTG-3′	5′-AGCCAGTGCCTCCTCATAAA-3′
EH047746	Helix-loop-helix protein (bHLH)	*Lotus japonicus*	2e-22	151	1.02 ± 0.006	5′-GTGGCTACGCTGAAAAACGAT-3′	5′-ATCCACATGGAGCTGTTTCC-3′
EH047058	Ubiquitin-protein ligase (U-BOX)	*Glycine max*	3e-58	192	1.03 ± 0.005	5′-GGTGCACCTTGAATGCTTCT-3′	5′-ATTCAATGCATCTCCGGTTC-3′
EH042075	Lipocalin (LIP)	*Solanum lycopersicum*	2e-114	195	1.06 ± 0.005	5′-CGTGTCTCAATGAGACTTGGA-3′	5′-TGGCCAATGAGAGCATACTG-3′
EH044012	DC1 domain-containing protein (DC1)	*Medicago truncatula*	3e-39	151	1.05 ± 0.007	5′-TGCAGGTATGTCCTCCATGA-3′	5′-GCACGTCCATTCTACAAGCA-3′
EH047773	Patatin-like protein (PN)	*Vigna unguiculata*	7e-56	206	0.91 ± 0.006	5′-AGCTGCACCCACATATCTCC-3′	5′-GCAGTGTCATCTGCTCTGTCCA-3′
EH046551	Transmembrane transporter (TMT)	*Arabidopsis thaliana*	1e-63	155	1.02 ± 0.006	5′-GCAGACGTCATTTTCTTTGG-3′	5′-TGATGATCGGTCAGGACAAA-3′
EH042384	Catalase (CAT)	*Cicer arietinum*	2e-22	153	1.01 ± 0.006	5′-TGAGGCATTGTCTGACCAAC-3′	5′-TGACCCCTTCTTCTTCCACA-3′
EH043050	Xyloglucan endotransglycosylase (XET)	*Medicago truncatula*	7e-106	101	1.05 ± 0.006	5′-CCATTTCCGAAGAATCAAGC-3′	5′-GCTTGGGTCCAATCAGTCTT-3′
JR330501	Integrin like (INT)	*Glycine max*	3e-45	168	1.04 ± 0.006	5′-AGCTTTCTCAGCCTCACCAA-3′	5′-GGAGGCATCAATAGCTTCCA-3′
GW276032	Phosphate-induced protein 1 (PHI1)	*Medicago truncatula*	2e-37	135	1.01 ± 0.006	5′-AGCTGTTAGCACCACGTTGATTGC-3′	5′-AAGCTTGGCATTTCACTAGGCACC-3′
GW276025	Tetraspanin-LEL-like (TET)	*Glycine max*	4e-78	198	1.02 ± 0.006	5′-AAGCATCACAGTCAGGATCGGTGT-3′	5′-TGCAATCTGGCAAACTCTGCTCTG-3′
EH047263	Auxin-repressed protein (ARP)	*Arachis hypogaea*	1e-50	60	0.99 ± 0.004	5′-GAGACAAGGAGCAAGCACCG-3′	5′-GCAAGACCTTGACCACGGAAG-3′
EH043626	Cytokinin dehydrogenase 3-like (CKX)	*Glycine max*	3e-111	110	1.04 ± 0.005	5′-TGACGGGATAGTGGTGAACA-3′	5′-GCCCACGTCAGCATAAGAAT-3′

products amplified from genomic DNA and cDNA templates in other *Arachis* species (data not shown), suggesting their broader application for the genus. RT-PCR provides a very sensitive method for detecting very small amounts of DNA contamination in RNA or cDNA samples and is a crucial step to assure sample purity and quality and to avoid false-positive results in qRT-PCR assays (Taylor *et al.* 2010).

Gene expression profiles

From the 19 selected genes related to plant response against root-knot nematodes (Table 1), 17 showed homology in BlastX to *A. stenosperma* sequences in transcripts database resources, for which primers were designed. Two genes, *AsPHI1* and *AsTET*, had no similarities identified in the *A. stenosperma* database and therefore previously described *A. hypogaea* primers were used (Tirumalaraju *et al.* 2011). The transferability of these primers among *Arachis* species was supported by their similarity to other wild species transcript sequences, including *Arachis ipaënsis* Krapov. & W. C. Greg. and *Arachis duranensis* Krapov. & W. C. Greg.

The PCR Miner algorithm was used to evaluate the amplification efficiency from each fluorescence raw data as an input (Zhao and Fernald 2005). This statistical algorithm, which does not require the establishment of a standard curve, proved to be fast and simple to use, as previously suggested (Marum *et al.* 2012). All primer pairs showed high efficiency, ranging from 0.91 to 1.06 (Table 1), and were used to adjust Cq values in subsequent analyses. The melt curve analysis supported the specificity of amplification of all transcripts as a single amplicon and no primer dimers were observed.

AsGAPDH and *As60S* were chosen as reference genes for qRT-PCR as they were established as the most stably expressed genes in roots of *A. stenosperma* subjected to biotic stress (Morgante *et al.* 2011). Comparison of absolute expression (Cq) values in all samples resulted in very similar expression levels, with *AsGAPDH* median= 23.08, quartiles 22.14–23.74, and *As60S* median = 23.28, quartiles 22.68–23.96. This indicated their stability along the assay time-course in both challenged and control *A. stenosperma* plants. These data corroborate our previous conclusions that these two genes are adequate for normalisation (Morgante *et al.* 2011), and adhere to the recommended use of more than one reference gene in qPCR analysis (Taylor *et al.* 2010).

Relative expression analysis of the 19 candidate genes within fold changing from –4.72 to 17.84 and their corresponding profiles are represented as a heat map (Fig. 1). The global expression pattern indicated a downregulation for the majority (58%) of the analysed genes at 3 DAI. However, throughout the assay, an increase in expression was observed, and 79% of the genes were upregulated at 9 DAI. These results agree with the expression profiles reported during root-knot nematode infection in *Arachis* (Guimarães *et al.* 2010; Tirumalaraju *et al.* 2011) and other plants, such as rice (Kyndt *et al.* 2012), *Arabidopsis* (Jammes *et al.* 2005; Barcala *et al.* 2010), tomato (Portillo *et al.* 2013) and cowpea (Das *et al.* 2010). These studies reported gene repression or constant expression at early stages of nematode–plant interaction, followed by a trend towards an increasing number of upregulated genes in both compatible and
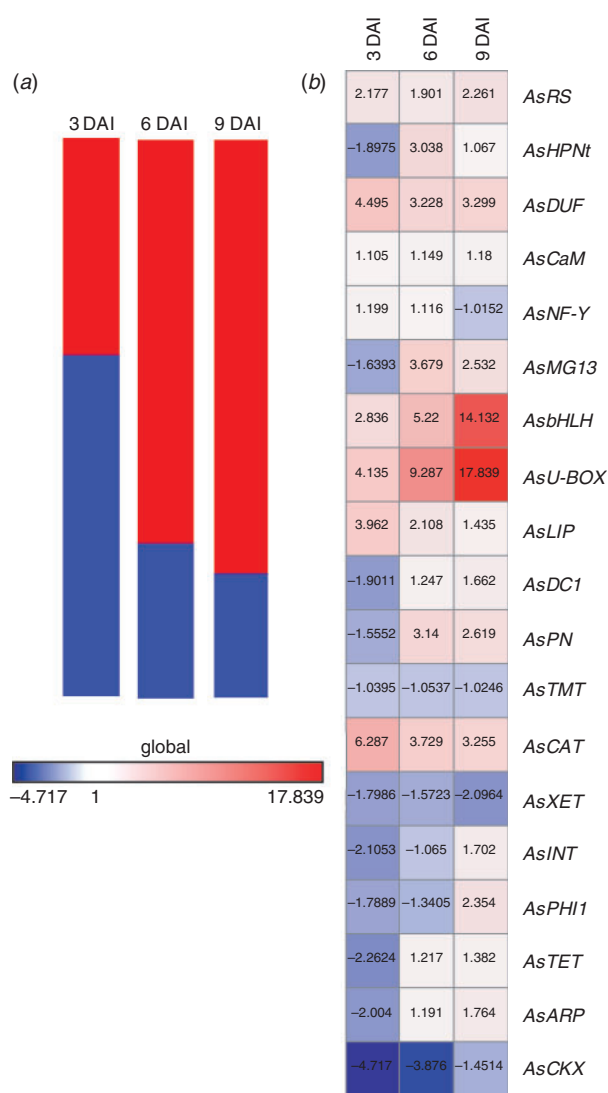

Fig. 1. Heat map of *Arachis stenosperma* gene expression values at three time points 3, 6, and 9 days after inoculation (DAI), during a plant–nematode interaction. (*a*) An overview of the percentage of up- and downregulated genes. (*b*) Normalised values, relative to day zero, are shown in a red–blue scale, the darker red being the most upregulated and the darker blue the most downregulated genes. Genes analysed: *AsRS*, resveratrol synthase; *ASHPNt*, hypothetical protein; *AsDUF*, DUF538; *AsCaM*, calmodulin; *AsNF-Y*, nuclear factor Y; *AsMG13* resistance protein MG13; *AsbHLH*, helix-loop-helix protein; *AsU-BOX*, ubiquitin-protein ligase; *AsLIP*, lipocalin; *AsDC1*, DC1 domain-containing protein; *AsPN*, patatin-like protein; *AsTMT*, transmembrane transporter; *AsCAT*, catalase; *AsXET*, xyloglucan endotransglycosylase; *AsINT*, integrin like; *AsPHI1*, phosphate-induced protein 1; *AsTET*, tetraspanin-LEL-like; *AsARP*, auxin-repressed protein; *AsCKX*, cytokinin dehydrogenase 3-like.

incompatible interactions. However, only a few differentially expressed genes involved in defence/stress responses were shared between both interactions. In our study, the selected 19 genes related to defence/stress showed modulation along the time points studied, which might play an important role in the defence response during incompatible *M. arenaria–A. stenosperma* interaction, leading to the HR-mediated cell death process.

Expression analysis of candidate genes

Of the 19 genes analyse, 16 showed significant differential expression in *A. stenosperma* roots infected with *M. arenaria* race 1 in at least one of the time points, seven genes being differentially expressed in all three time points analysed (Fig. 2). With the exception of *AsPHI1*, all genes showed significant differential expression at 3 DAI, suggesting an extensive transcriptional regulation in the early stages of nematode infection. At 6 and 9 DAI, almost half of the genes showed significant diverse expression profiles.

An upregulation of ~2-fold at all time points was observed for *AsRS2*, *AsbHLH*, *AsU-BOX*, *AsDUF*, and *AsCAT* in inoculated plants, relative to day 0 (Figs 1, 2). The most upregulated genes were those encoding bHLH and U-BOX, with maximum fold values of 14.13- and 17.84-, respectively, at 9 DAI (Fig. 1). The most downregulated gene was *AsCKX*, showing lower values at early stages of nematode infection (3 and 6 DAI).

AsARP and *AsCKX* sequences were further used as probes for *in situ* hybridisation to determine the spatial distribution of its transcripts in the cells of infected and control roots during initial phases of the nematode interaction. The presence of RNA in different cells as indicated by the redish/orangish colors (Fig. 3*a*) was confirmed in the acridine stained sections of all samples. *AsARP* transcripts were detected in cortical and xylem cells of both infected and non-infected *A. stenosperma* roots, collected

at 3, 6 and 9 DAI (Fig. 3*b–d*). We noted that hybridisation signals were clearly stronger and more dispersed in inoculated than control roots in these same tissues. No signals could be detected in epidermal or phloem cells. When *AsCKX* probes were used for *in situ* hybridisation analysis in *A. stenosperma* control roots, weak signals of *AsCKX* transcripts could be detected with the anti-sense probe, whilst the sense probe lacked signal. On the other hand, in inoculated *A. stenosperma* roots probe signal was weaker than in the control (Fig. 3*e–g*), confirming the low expression of this gene as indicated by qRT-PCR.

Discussion

Worldwide, *M. arenaria* race 1 is the most important nematode constraint to peanut that shows limited resistance to this parasite, with most cultivars (e.g. COAN and Nematan) relying on a single resistance source from *A. cardenasii*. In previous studies, Proite *et al*. (2008) showed that the resistance harboured by the wild peanut relative *A. stenosperma* was due to a HR-mediated cell death process, which is characterised by the rapid development of cell death immediately surrounding infection sites (Morel and Dangl 1997).

In order to identify and characterise genes involved in this incompatible interaction, the transcriptional profile of 19 candidate genes potentially involved in the resistance response were analysed in the early stages of the infection.
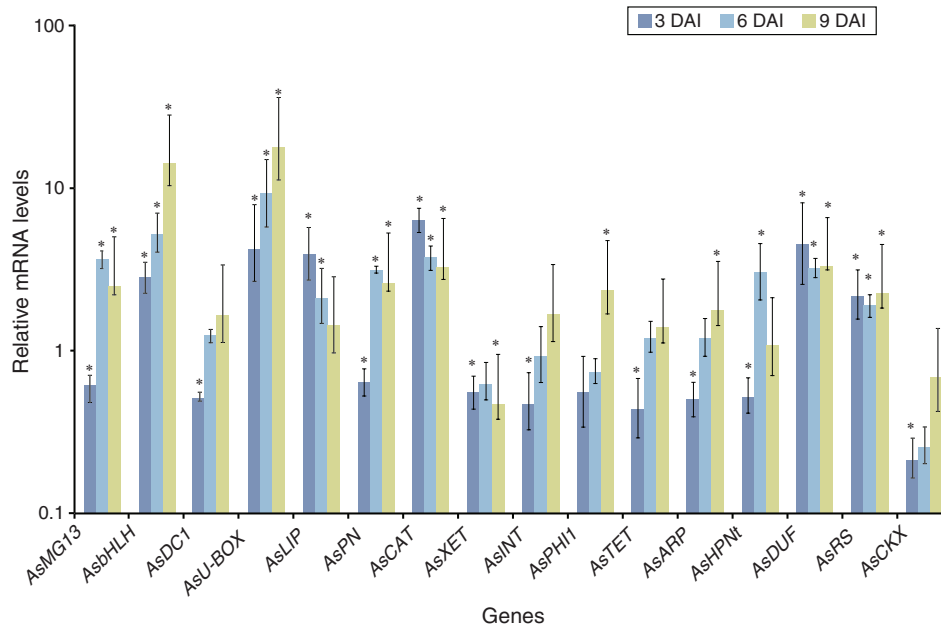

Fig. 2. Relative mRNA levels of 16 candidate-genes in *Arachis stenosperma* roots inoculated with *Meloidogyne arenaria* at 3, 6 and 9 days after inoculation (DAI), relative to day 0. Bars represent the standard error of the mean of two biological replicates for each sample. Significantly up- or downregulated genes are indicated: *, $P < 0.005$. Genes analysed: *AsRS*, resveratrol synthase; *AsHP*, hypothetical protein; *AsDUF*, DUF538; *AsCaM*, calmodulin; *AsNF-Y*, nuclear factor Y; *AsMG13* resistance protein MG13; *AsbHLH*, helix-loop-helix protein; U-BOX, ubiquitin-protein ligase; *AsLIP*, lipocalin; *AsDC1*, DC1 domain-containing protein; *AsPN*, patatin-like protein; *AsTMT*, transmembrane transporter; *AsCAT*, catalase; *AsXET*, xyloglucan endotransglycosylase; *AsINT*, integrin like; *AsPHI1*, phosphate-induced protein 1; *AsTET*, tetraspanin-LEL-like; *AsARP*, auxin-repressed protein; *AsCKX*, cytokinin dehydrogenase 3-like.
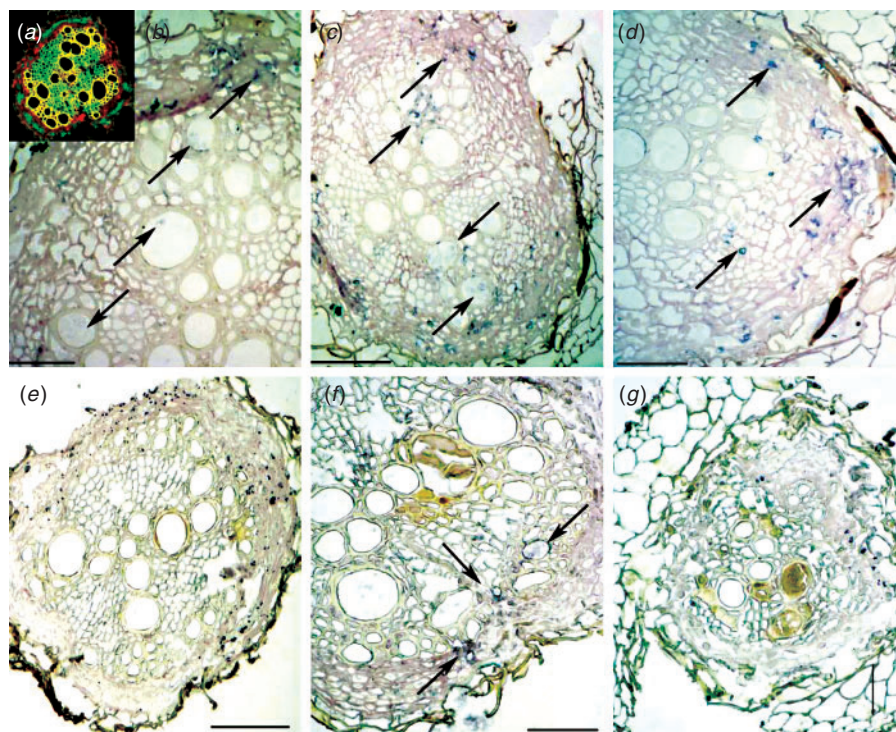
Fig. 3. Butyl-methyl methacrylate semi thin sections of *Arachis stenosperma* roots inoculated or non-inoculated with *Meloidogyne arenaria* at three time points, 3, 6, and 9 days after inoculation (DAI). (*a*) Section of inoculated root at 9 DAI treated with acridine orange, confirming the preservation of RNA after sample preparation, as indicated by the redish/orangish colors in different cells. Scale bar indicates 100 μm. (*b*) Non-inoculated root collected at 3 DAI, hybridised with anti-sense *AsARP* probe showing signals in cortical and xylem cells (arrows). No signals were detected in epidermal or phloem cells. Scale bar corresponds to 50 μm. (*c, d*) Inoculated root collected at 6 DAI (*c*) and 9 DAI (*d*) hybridised with *AsARP* anti sense probe showing stronger and more dispersed hybridisation signals in cortical and xylem cells (arrows). No signals were detected in epidermal or phloem cells. Scale bars correspond to 50 and 100 μm respectively. (*e*) Inoculated root collected at 6 DAI hybridised with *AsCKK* sense probe, with no detectable hybridisation signals. Scale bar corresponds to 100 μm. (*f*) Non-inoculated root collected at 3 DAI hybridised with anti-sense *AsCKK* probe showing weak signals (arrows). Scale bar corresponds to 50 μm. (*g*) Inoculated root collected at 3 DAI hybridised with *AsCKK* anti-sense probe that yielded no signals. Scale bar corresponds to 100 μm.

The 16 differentially expressed genes during early stages of an *A. stenosperma*–*M. arenaria* race 1 interaction identified were placed into five general groups, on the basis of their GO annotation (http://www.blast2go.com/b2ghome, accessed 11 July 2012), and role in plant-pathogen interaction: resistance (R) genes, transcriptional regulation, pathogen defence response, membrane/cell wall components, and hormonal balance. In those groups we found genes involved in HR and production of secondary metabolites related to pathogen defence.

R genes

Hypersensitive response can be triggered by a wide variety of pathogens and occurs within a few hours following pathogen contact (Meyers *et al.* 2005). In some cases this response is conditioned by the presence in the pathogen of an avirulence (*avr*) gene, the product of which is recognised by a plant possessing the corresponding R gene. This mechanism was described by Flor (1946) as the 'gene-for-gene' hypothesis. In other cases, when the Guard Model (Dangl and Jones 2001) is

applied, pathogen effectors (pathogen-secreted proteins that manipulate host cell functions), are indirectly perceived by R proteins, which trigger disease resistance in the host. This model explains how multiple effectors could be recognised by a single R protein, enabling a small number of R genes to target a broad diversity of pathogens.

In the present study, an *A. stenosperma* homolog of soybean *MG13* (*AsMG13*) was upregulated at 6 and 9 DAI in roots of infected plants. This gene was first identified in cDNA libraries of soybean roots infected with the cyst nematode *Heterodera glycines* as containing conserved R gene domains, belonging to the NBS-LRR family (Graham *et al.* 2000). Such plant disease R genes mediate specific pathogen recognition, often leading to successful immune response. Downstream responses of R genes include ion fluxes, oxidative burst, rapid release of reactive oxygen species (ROS), transcriptional reprogramming and, in many cases, HR causing cell death at the infection site (Tornero *et al.* 2002). Several sedentary nematode R genes (*Mi-1*, *Hero A*, *Gpa2* and *Gro1-4*) resemble other plant R genes, falling into the NBS-LRR class (Williamson and Kumar 2006). Considering the

compatible timing of the occurrence of the resistance-mediated by HR in *A. stenosperma* (8 DAI) (Proite *et al.* 2008) and the herein transcription profile of *AsMG13*, we hypothesise that this gene is involved in pathogen recognition that triggers the resistance response to *M. arenaria*.

Transcriptional regulation

Following the pathogen recognition step by R genes, signal transduction pathways are triggered, often leading to the induction of defence-related genes. Some of these TFs are specifically induced by a plant–pathogen interaction; however, abiotic factors and developmental events can also be a trigger (Morel and Dangl 1997).

Basic helix-loop-helix (bHLH) proteins constitute one of the largest TFs families in *Arabidopsis* (Heim *et al.* 2003). In this study, the significant upregulation of an *A. stenosperma* homolog (*AsbHLH*) in infected roots corroborates previous macroarray data (Guimarães *et al.* 2010). Some of these bHLH TFs form clusters regulating specific jasmonic acid (JA)-dependent responses, which modulate processes including defence against pathogens (Fernandez-Calvo *et al.* 2011). JA, a stress-induced hormone, is involved in defence against insects, pathogens and abiotic stresses, and can also induce the production of secondary metabolites, including alkaloids, anthocyanins, and terpenoid compounds (Niu *et al.* 2011). The increase in ROS levels in the cells and the induction of the synthesis of secondary metabolites like phythoalexins cause cell death (Chang *et al.* 2011). We suggest that the upregulation of *AsbHLH* during *A. stenosperma* response to *M. arenaria* infection induces the production of JA and therefore contributes to the HR response. Another TF analysed here, *AsDC1*, previously reported to be induced by cell wall microbe components (Shinya *et al.* 2007), showed an expression profile that did not correlate to the development of the defence response.

Pathogen defence response

One of the outcomes of the defence related intracellular signalling is the activation of defence genes. Seven genes involved in the synthesis of anti-microbial compounds and pathogenesis-related (PR) proteins were found to be differentially regulated in this study, as discussed below.

Patatins are glycoproteins with a non-specific lipid acyl hydrolase activity that can initiate the synthesis of fatty-acid-derived defence signals against stress (Wang 2004; Yang *et al.* 2007). The upregulation of patatin-like genes has been associated to defence and triggering of cell death and HR upon infection of a diverse range of pathogens (Dhondt *et al.* 2000, Cacas *et al.* 2009; Orlowska *et al.* 2012), including *M. arenaria* race 1 in *A. hypogaea* roots (Tirumalaraju *et al.* 2011). Likewise, we found upregulation of *AsPN* expression in roots infected with *M. arenaria* at 6 and 9 DAI, leading us to consider its association in the promotion of the HR response, through JA production, as previously described in *Arabidopsis* (Yang *et al.* 2007).

Another protein related to defence is resveratrol synthase (RS), the last of the four enzymes that participate in the resveratrol biosynthesis, which can be induced by different biotic and abiotic stresses (Chang *et al.* 2011). Indeed, the accumulation of RS and its mRNA was observed in peanut in response to treatment with stress hormone, yeast extract, ultraviolet light, and after wounding (Chung *et al.* 2003), fungal chitin treatment (Yang *et al.* 2010), and infection with *Aspergillus* spp. (Sobolev 2008). Recently, it was shown in *Vitis vinifera* that resveratrol, in addition to its classical role as an antimicrobial phytoalexin, is an important regulator for initiation of HR-related cell death (Chang *et al.* 2011). In the present study, a variant homolog of *A. stenosperma* RS gene *AsRS1*, previously analysed using macroarray by Guimarães *et al.* (2010), named *AsRS2*, showed strong upregulation in the three time points analysed, coinciding with the occurrence of HR in *A. stenosperma*. Therefore, we suggest that *AsRS2* is involved in the HR response of *A. stenosperma* to *M. arenaria* and is one of our premium candidates for overexpression in transgenic peanut to enhance nematode resistance.

Several transcriptome studies have demonstrated the upregulation of *PHI1* (*Phosphate-induced protein 1* gene) in resistant plants in response to pathogen attack, including nematodes (Fosu-Nyarko *et al.* 2009), virus (Hamada *et al.* 2008) and bacteria (Kottapalli *et al.* 2007). It seems that this protein has a common role in the response to diverse pathogens and could be triggering the HR. In the present study, the upregulation of *AsPHI1* at 9 DAI in nematode-inoculated roots of *A. stenosperma* coincided with the previously demonstrated appearance of HR (Proite *et al.* 2008). Another defence-related protein, lipocalin (*AsLIP*) showed gradual decline of expression during nematode infection. This suggests that this lipocalin homolog might act as a scavenger for the products of the oxidative stress during HR (Charron *et al.* 2008).

Likewise, plant genes containing the U-BOX (PUB) domain have been demonstrated to be upregulated under pathogen infection (Yee and Goring 2009) or after elicitor treatment, such as chitin, found in nematode exoskeleton (Libault *et al.* 2007). The silencing of ubiquitin-related genes reduced the HR in tobacco and pepper, whereas its over-expression enhanced disease resistance in *Arabidopsis* (González-Lamothe *et al.* 2006; Lee *et al.* 2011). Here, we identified a PUB (*AsU-BOX*) gene in roots of *A. stenosperma*, which was the gene most upregulated during the early stages of infection. This gene showed a gradual and significant increase in its relative expression along the bioassay time course, from 4-fold at 3 DAI to 17-fold at 9 DAI (Fig. 1). These results are in accordance with our previous macroarray analysis (Guimarães *et al.* 2010), reinforcing the important role that this protein may exert in mediating the HR.

Another protein family containing a specific domain related to plant defence is the DUF protein family, identified in *Arabidopsis*, rice and tomato plants grown under various environmental stress conditions, such as nutrient deficiency, *Agrobacterium*-induced crown gall, and mixed elicitors (Brunings *et al.* 2009; Gholizadeh 2011). DUF protein was shown to elevate activity of enzymes that induce the HR response, such as catalase, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase, when applied to tobacco leaves (Gholizadeh 2011). In this study, *AsDUF* was upregulated in the three time points studied, suggesting its role in the induction of these ROS enzymes. Catalases play an important role in the detoxification of ROS in the cell, and its inhibition allows

hydrogen peroxide level to be increased, thus, triggering HR (Molinari 1998). The upregulation of *AsCAT* that we observed in the three time points analysed was compatible with the occurrence of the HR and consequently cell death around 9 DAI, as previously described in *A. stenosperma* (Proite *et al.* 2008).

Membrane/cell wall components

Upon root-knot nematode infection, host cells are transformed into multinucleated feeding sites – the giant cells. The molecular mechanisms underlying this reprogramming of root cell fate to acquire functions that benefit nematodes include cell wall modification and extensions by proteins such as expansin, integrins, tetraspanin and xyloglucan endotransglycosylase (Davis and Mitchum 2005; Wieczorek and Seifert 2012).

The expression profiles of two genes encoding interacting plasma membrane proteins, an integrin and a tetraspanin, involved in cell adhesion and signal transduction (Bassani and Cingolani 2012), were also investigated in this study. The role of these two proteins in cell signalling and plasma membrane–cell wall junction integrity in the root-knot nematode giant cell formation involves a restructuring of plasma membrane and cell wall continuum. We showed that both genes exhibited the same expression profile, being downregulated at 3 DAI, but with no significant differential expression afterwards. These results are in agreement with Tirumalaraju *et al.* (2011), who showed that there was no increase expression of these genes in the peanut nematode resistant cultivar Nematam, but an upregulation in the susceptible cultivar Florunner. Similarly, we observed a *xyloglucan endotransglycosylase* homolog (*AsXET*) was downregulated at all the three time points. Therefore, we hypothesise that the low expression of these three genes in the early stages of nematode infection in the resistant *A. stenosperma* could interfere with the cell wall loosening and elongation process necessary for gall formation.

Hormonal balance

Endoparasitic nematodes induce hormonal changes in the host, contributing to the formation of giant cells. Auxin signalling is essential for the formation and maintenance of the root-gall (reviewed by Goverse *et al.* 2000; Bird and Kaloshian 2003). However, less is known about genes that are downregulated by auxin, such as auxin repressed protein gene (*ARP*), with some of them related to biotic and abiotic stresses and plant development (Song *et al.* 2007; Salvianti *et al.* 2008).

Previous analyses by macroarray and northern blotting showed that *AsARP* was differentially expressed between infected and control roots of *A. stenosperma* and also in susceptible *A. hypogaea* roots (Guimarães *et al.* 2010). Our present data corroborate the expression profile of *AsARP* in *A. stenosperma*. In qRT-PCR analysis, *AsARP* showed initial downregulation at 3 DAI followed by a gradual increase in expression as nematode infection progressed. Considering that the *AsARP* expression is inversely related to the accumulation of auxin, our data are compatible with a similar study in soybean, suggesting that after an initial auxin increase in the developing giant-cells, root-knot nematodes decrease auxin biosynthesis in feeding cells at later stages of the infection, thus, altering plant cell development (Doyle and Lambert 2003). *AsARP* transcripts were detected by *in situ* hybridisation in cortical and xylem cells and, after gene upregulation, an increase in the concentration of transcripts was observed in these same cells. This confirms the specific localisation of these transcripts in cells that are largely involved in water transportation into the central cylinder of the root, mostly through diffusion.

In parallel to auxins, cytokinins also have been shown to play an important role in root-knot nematode gall formation, with high levels of expression induced in the early stages of plant-parasite interaction (Goverse *et al.* 2000; Barcala *et al.* 2010). *Cytokinin dehydrogenase* (*CKX*) expression is induced by cytokinins, representing a negative feedback system, in which the accumulation of cytokinin induces its own catabolism, controlling the levels of the hormone in the cell (Lee *et al.* 2007). Lohar *et al.* (2004) showed that *CKX* overexpression in *Lotus* hairy roots reduced gall formation, indicating that the initial stages of gall cell must require high levels of cytokinins.

In our study, *AsCKX* clearly showed, by both qRT-PCR and *in situ* hybridisation, a diminishing expression during the first days of nematode infection, especially at 3 and 6 DAI. This corroborated our previous macroarray and northern blotting data that showed *AsCKX* downregulation, especially in contrast with the susceptible *A. hypogaea* (Guimarães *et al.* 2010). We suggest that, as in *A. stenosperma*, there was no induction of *AsCKX* because the feeding site was not established due to HR, and, as previously shown, there was no increase in the influx of cytokinin in the cells surrounding the nematode feeding site (Lohar *et al.* 2004).

Conclusively, *in situ* data correlated with those obtained by qRT-PCR for the two hormonal balance related genes (*AsARP* and *AsCKX*) studied during the early phases of the infection. Their involvement in the differentiation of plant cells into nematode feeding sites will be studied further in plant models to evaluate their potential as candidate genes for peanut transformation and nematode resistance improvement.

Conclusion

The modulation of most gene expressions analysed here is in accordance with the occurrence of the HR, which is the resistance mechanism employed by *A. stenosperma*. The fact that genes with differential expression profiles representing different steps of the defence response-recognition pathogen signal, intracellular signalling, transcriptional activation, cell wall and membrane modification, hormone rebalance and production of secondary metabolites, reassures the functionality of the resistance network.

To validate these gene functions and understand their role in the HR response of *A. stenosperma* to *M. arenaria* race 1 interaction, they will be over-expressed/silenced in model plants (*Medicago trunculata* and *Arabidopsis*) or in composite peanut plants with transgenic *Agrobacterium rhizogenes* roots. The built up knowledge in these candidate genes is of great interest for engineering nematode resistance in peanut.

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