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Genome-wide identification and expression analysis of Dormancy-Associated gene 1/ Auxin Repressed protein (DRM1/ARP) gene family in *Glycine max*

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ABSTRACT

Dormancy-Associated gene 1/Auxin Repressed protein (DRM1/ARP) genes are responsive to hormones involved in defense response to biotic stress, such as salicylic acid (SA) and methyl jasmonate (MeJA), as well as to hormones that regulate plant growth and development, including auxins. These characteristics suggest that this gene family may be an important link between the response to pathogens and plant growth and development. In this investigation, the DRM1/ARP genes were identified in the genome of four legume species. The deduced proteins were separated into three distinct groups, according to their sequence conservation. The expression profile of soybean genes from each group was measured in different organs, after treatment with auxin and MeJA and in response to the nematode *Meloidogyne javanica*. The results demonstrated that this soybean gene family is predominantly expressed in root. The time auxin takes to alter DRM1/ARP expression suggests that these genes can be classified as a late response to auxin. Nevertheless, only the groups 1 and 3 are induced in roots infected by *M. javanica* and only group 3 is induced by MeJA, which indicates a high level of complexity in expression control mechanisms of DRM1/ARP family in soybean.

Keywords: auxin; methyl jasmonate; gene expression; plant–pathogen interaction.

1. Introduction

Auxins are an important class of plant hormones that orchestrate plant growth and development through gene expression regulation (Abel and Theologis, 1996; Verhage *et al.*, 2010;). Some auxin responsive genes were described and all of them are involved in some aspects with plant growth and development. The auxin responsive genes include small auxin up RNA (SAUR), Gretchen Hagen3 (Gh3), Auxin/Indole-3-AceticAcid (Aux/IAA), auxin responsive factors (ARF), and dormancy-associated gene 1 (DRM1)/auxin repressed protein (ARP) (Hagen and Guilfoyle, 2002; Kim *et al.*, 2007).

The DRM1/ARP family is exclusive to and conserved in higher plants (Park and Han, 2003). Gene expression analyses indicated that these proteins probably present a role during plant development and growth (Park and Han, 2003; Wood *et al.*, 2013). However, the biochemical function of DRM1/ARPs proteins have not been

characterized yet. The initial characterization of DRM1/ARP family demonstrated that the orthologous genes PsDRM1 and PsDRM2 from *P. sativum* are expressed in dormant axillary buds. Similar results are found in *Arabidopsis*, sorghum (*Sorghum bicolor*) and kiwi (*Actinidia deliciosa*) (Kebrom *et al.*, 2010; Stafstrom *et al.*, 1998; Tatematsu *et al.*, 2005; Wood *et al.*, 2013). The expression of DRM1/ARP from *P. sativum* and *B. rapa* occurs in mature tissues or tissues where growth has ceased, which indicates that their functions may be related to growth interruption (Lee *et al.*, 2013; Stafstrom *et al.*, 1998). The expression of BrARP1 and BrDRM1 was inversely correlated with the expression of the growth marker BrRPL27 in many tissues (Lee *et al.*, 2013). Similar results were observed in axillary buds of kiwi for Ade-DRM1 and Ade-CDKB genes (Wood *et al.*, 2013).

Some members of the DRM1/ARP family were analyzed in response to biotic stress (Kim *et al.*, 2007; Zhao *et al.*, 2014; Poupin *et al.*, 2016). The silencing of GERI/ARP1 increased plant susceptibility to infection by *Tobacco mosaic virus*, *Pectobacterium carotovorum* subsp. *carotovora*, and *Phytophthora parasitica* var. *nicotianae*. A similar result was observed for the TaARP gene from *Triticum aestivum*. The knockdown of TaARP resulted in higher susceptibility to fungus *Blumeria graminis* f. sp. *tritici*. (Song *et al.*, 2014; Zhao *et al.*, 2014). The NbARP1 gene is expressed in *Nicotiana benthamiana* infected with *Hibiscus latente* singarope virus (HLSV) and *Tabacco mosaic virus* (TMV) (Salvianti *et al.*, 2008). EuNOD-ARP1 is mainly expressed in root nodules of *Elaeagnus umbellata* infected with the symbiont fungus *Frankia*, compared to the uninfected organ (Kim *et al.*, 2007). AtDRM2 is induced in response to infection by the fungus *Trichoderma harzianum*, and the rhizobacteria *Burkholderia phytofirmans* PsJN (Morán-Diez *et al.*, 2012; Poupin *et al.*, 2016).

Moreover, the DRM1/ARPs genes are induced in incompatible interactions between peanut plants and the peanut root knot nematode, *Meloidogyne arenaria* (Proite *et al.*, 2007; Guimarães *et al.*, 2010). For *Glycine max*, a DRM1/ARP gene is expressed during the initial phase of incompatible interactions. Two DRM1/ARP genes were differentially expressed in response to nematode *Meloidogyne javanica*. It provides insights into the role of ARP genes in plant stress responses, especially in nematode- interactions (De Sá *et al.*, 2012).

DRM1/ARP genes are responsive to hormones involved in defense response to biotic stress, such as salicylic acid (SA), besides hormones that control growth and development, such as ethylene and auxins. These characteristics suggest that this gene family may be an important link between the response to pathogens and plant growth and development. Nevertheless, the transcriptional profile of DRM1/ARP genes in relation to auxin remains controversial. Auxin reduces SAR5 mRNA accumulation in strawberry during fruit development (Reddy and Poovaiah, 1990). The RpARP transcript levels in *Robinia pseudoacacia* are completely extinguished 6 hours after treatment with exogenous auxin (Park and Han, 2003). AtDRM1 and AtDRM2 transcripts are reduced in response to IAA (Rae *et al.*, 2014). On the other hand, PpARP1 and PpARP2 genes are induced by 0.2mM of IAA in *Pyrus pyrifolia* (Shi *et al.*, 2013). In *Brassica napus*, auxin 2,4-Dichlorophenoxyacetic acid strongly induces the accumulation of BnARP1 transcripts after 2 hours of treatment in transgenic lines of *Arabidopsis* overexpressing BnARP1 (Wu *et al.*, 2017).

The availability of the complete soybean genome sequence allows more detailed studies on gene families that may play a role in plant defense pathways. Since the DRM1/ARP family has not been characterized in any legume species, in this work, DRM1/ARPs genes were identified in the genome of four legume species and clustered according to the phylogenetic relationship and sequence conservation. The expression profile of each defined group was measured in four soybean organs, after treatment with auxin and MeJA hormones, and in response to the nematode *M. javanica*.

2. Materials and Methods

2.1. Identification of DRM1/ARP genes in four legume genomes

The AtDRM1 (Gene ID: At1g28330) amino acid sequence was used as query on BLASTP algorithm (Altschul *et al.*, 1990) and searched against the predicted proteins based on genome sequence from four legume genomes: *Glycine max* (Gm), *Medicago truncatula* (Mt), *Phaseolus vulgaris* (Pv) and *Trifolium pratense* (Tp), retrieved from Phytozome release v12.1.5 (Goodstein *et al.*, 2012). In addition, the search was extended to two species from the *Brassicaceae* family (*Brassica rapa* and *Arabidopsis thaliana*). A cutoff e-value of 10×10^{-10} was defined for all searches. The presence of a typical *dormancy/auxin associated protein domain* in all recovered sequences was

confirmed using the Pfam database release 31.0 (Finn *et al.*, 2014). The theoretical isoelectric point and molecular weight of the amino acid sequences were calculated using the ExPASy Compute pI/Mw tool (Artimo *et al.*, 2012). The gene IDs, protein length and other- information are summarized in the Supplementary Table 1.

2.2. Legume DRM1/ARP cluster analyses

The protein sequences were aligned using Clustal Omega software system with default parameters (Sievers and Higgins, 2014). The alignment was used to calculate a pair-wise identity matrix. The pair-wise identities were used to calculate the distances between the sequences using the Phylip software (Felsenstein, 1989). A visual representation of the distance matrix was obtained by multidimensional scaling (MDS) plot with two dimensions (2D). The cluster identification in the plot was performed by the K-means method (Steinley, 2006). The MDS, clustering, and plot were performed using the R software platform (R Development Core Team, 2011).

2.3. Sequence alignment and phylogenetic analyses of DRM1/ARP genes

The amino acid sequence of DRM1/ARP from *A. thalina*, *B. rapa* and four legumes (Gm, Pv, Tp, Mt) present in each cluster identified by K-means analyses were used to create phylogenetic trees on MEGA 7.0 (Kumar *et al.*, 2016). The phylogenetic trees were built using the neighbor-joining method with the Kimura 2-parameter model and uniform rates among the sites. The tree quality was estimated using bootstrap resampling with 1,000 replications.

2.4. Primer design of specific groups of DRM1/ARP in soybean

The nucleotide coding sequences of each cluster were aligned using the on-line Clustal Omega program (Sievers and Higgins, 2014). Conserved regions between sequences from the same cluster and polymorphic sequences of other clusters, both smaller than 200pb, were selected among all the sequences for primer design (Cao *et al.*, 2016; Freitas *et al.*, 2011; Dos Santos *et al.*, 2012). Primer sequences were evaluated for the formation of homodimers and heterodimers, using the Oligo Explorer software (Gene Link). Primer specificity was assessed using the Electronic PCR (e-PCR) software (Rotmistrovsky *et al.*, 2004) against soybean genome retrieved from

Phytozome release v12.1.5 (Goodstein *et al.*, 2012). Primers for reference genes were synthesized according to Le *et al.* (2012) and Miranda *et al.*, (2013) (Supplementary Table 1), that used and validated these genes as constitutive in their experiments in soybean.

2.5. Expression profile of DRM1/ARP genes in soybean organs and in response to phytohormones.

The expression of DRM1/ARP clusters was analyzed in leaf, stem, root (V3 stage) and mature seed of more than 450 mg of soybean cultivar CD 206 (Alves *et al.*, 2015). In order to evaluate gene expression in response to exogenous application of phytohormones, the soybean plants of cultivar CD 206 were grown in a greenhouse, and when they reached stage V3. To the auxin treatment, the leaves were collected and sanitized with water, alcohol, and distilled water. The detached leaves were incubated in the dark at 60 rpm at 25 ° C in petri dishes containing MS medium (1% sucrose, 0.5 g / L MES, 0.01% (v / v) Tween 20, pH 5.8) supplemented with and 100µM indole-3-acetic acid (Sigma-Aldrich) (Kim *et al.*, 2007). After one, six and twelve hours of treatment with auxin. The control corresponds to the time zero hours of the experiment and without supplementation with the hormone. Each biological sample represents a pool of leaves of three plants many different. To the JA treatment, V3 stage soybean plants were sprayed until dripping with a solution of 100 mM of MeJA (Sigma-Aldrich). Samples were collected 0, 1, 6, and 12h after exposure to the hormones (Alves *et al.*, 2015). After harvesting, the plant materials were immediately frozen in liquid nitrogen and stored at –80 °C until RNA extraction.

2.6. Infection of soybean plants using *Meloidoygne javanica* nematode

Ten-day-old plants of the susceptible soybean cultivar “Desafio” were inoculated with a solution composed by 20000 J2 *M. javanica* nematode mixed in 5mL of water. Root samples from inoculated plants and mock were harvested 3 days after inoculation. As control treatments, plants were grown under the same conditions and were subjected to false inoculation (MOCK). Then, each sample was carefully rinsed with water, immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction.

2.7. cDNA synthesis and qPCR data analysis

Total RNA was extracted from each sample using Trizol reagent (Invitrogen). After purification, the RNA was quantified by spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific) and analyzed on 1.5% (w/v) agarose gel. A total of 2 µg of RNA was treated with one unit of DNase I Amplification Grade (Sigma-Aldrich) to remove potential contamination with genomic DNA. The cDNA synthesis was performed using 2 µg of total RNA and High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). The qPCR reaction conditions were performed on a StepOne Real Time PCR platform (Applied Biosystems) using specific primers (Supplementary Table 2). The R^2 was calculated for each primer set, and the reaction efficiency was calculated using the following formula: $E = (10^{-1/\text{slope}}) - 1$. The amplification reactions were performed under the following conditions: 10 min at 95°C; 40 cycles of 15 s at 94°C and 1 min at 60°C. After the amplification step, a melting curve was performed for each primer set. The relative quantification was done by a standard curve obtained for each gene, by the equation of the line relating average Ct and log10 of control cDNA concentration (Freitas *et al.*, 2011; Dos Santos *et al.*, 2012). The results were normalized with *UBC4*, *CYP2*, and *ELF1A* references genes. The results were analyzed using ANOVA, and the graphs were prepared using the GraphPad Prism 5.0 software (GraphPad Inc.).

3. Results and discussion

3.1. DRM1/ARPs genes in four legume plant species

The amino acid sequence of AT1G28330.5 was used to obtain other DRM1/ARP family members in *A. thaliana*. All 13 sequences obtained from *A. thaliana* were individually used in a new screening in four legume species and *B. rapa*. Protein sequences with an e-value $\leq 1^{-10}$ were initially selected and the *dormancy/auxin associated protein* (PF05564) domain was confirmed using PFAM databases. The candidates without full domain were discarded from the study.

Through the analyses of sequence similarity and search for *dormancy/auxin associated protein* domain, 27 protein sequences were identified in Gm, 12 in Mt, 8 in Pv, 5 in Tp, 11 in Br and 13 in At. Despite to be a conserved family in different species

of plants, the gene number in each species varies. Soybean presented the largest DRM1/ARP family among the species investigated in this study. The 27 proteins are codified by 8 genes through the alternative splices and located in 8 different chromosomes, numbered as 3, 7, 10, 13, 15, 16, 19 and 20. This genetic family has few members in *A. thaliana* (5 genes) and Kiwi - *Actinidia deliciosa* - (8 genes) (Wood *et al.*, 2013; Rae *et al.*, 2014). The size of soybean predicted proteins ranging from 106 to 211 amino acids, with a molecular weight between 11.59 (Glyma.13G237000.3) and 22.51 kDa (Glyma.19G232400.3). DRM1/ARP proteins usually were small proteins, with molecular weight less than 20 kDa and characteristics of basics proteins (Wood *et al.*, 2013). All of them have a high theoretical isoelectric point, ranging from 8.89 (Glyma.13G237000.2) to 10.26 (Glyma.20G237200.3). Detailed information about these genes is provided in Supplementary Table 2. Results showed that DRM1/ARP is a gene family highly conserved in plant at the protein level, but more studies about its function are necessary (Wood *et al.*, 2013), principally in soybean.

3.2. Phylogenetic analysis and classification of legume DRM/ARP genes

The sequence similarity analysis and K-means clustering showed that the proteins from the four legumes, *A. thaliana* and *B. rapa* can be distributed into three well-defined clusters (Figure 1A). In order to analyze the phylogenetic relationship among the DRM1/ARP family, all protein sequences from each group were used to build phylogenetic trees on MEGA 7.0 (Figure 1 B, C and D). The presence of these three well-defined clusters suggests that the sequence-specificity of this family arose prior to legume speciation. Park and Han (2003) suggest that this family may have arisen before phylogenetic separation between monocots and dicots due to the high levels of conservation among the DRM1/ARP genes. Presumably, the pattern of conservation was maintained in these species due to their fundamental role in the growth and development of higher plants (Park and Han, 2003). However, the proteins Gm21, Gm22, Gm23, Gm24, Gm25, Gm26 and Gm27 from cluster two compose an exclusive soybean clade, which suggests that these genes have specific roles in soybean.

3.3. Expression analyses of soybean DRM/ARP genes in different soybean organs and in response to hormones

DRM1/ARP genes have been related to plant growth repression and bud dormancy maintenance (Lee *et al.*, 2013; Wood *et al.*, 2013, Zhao *et al.*, 2014; Rae *et al.*, 2014). However, the relationship between this family and growth and development in soybean has not been studied. Aiming to increase knowledge about the possible role of DRM1/ARP genes in soybean, the expression profile of this family was evaluated by qPCR in different organs of soybean plant (root, stem, leaf and mature seed). High expression was observed in the root, for all three groups (Figure 2). These results are corroborated by studies on *B. rapa* and *A. thaliana*. BrARP1 and BrDRM1 genes show high transcript levels in root, hypocotyl, mature leaf, petals and sepals (Lee *et al.*, 2013). Some alternative transcripts of the AtDRM1 and AtDRM2 genes exhibit a similar expression profile in many tissues, being relatively higher in non-growing tissue (Rae *et al.*, 2014).

The transcriptional profile of the DRM1/ARP family was analyzed after MeJa and IAA hormone treatment in soybean leaves. The expression of genes activated by auxin (AUX/IAA) (Ding *et al.*, 2008) or MeJa (VSP2) (Fernández-Calvo *et al.*, 2011) pathways was used to validate both hormone treatments (Figure 3 and Figure 4). All clusters of soybean DRM1/ARP protein were induced 12h after the auxin treatment, while MeJa affected the only Cluster 3 6h after the treatment (Figure 3 and Figure 4). DRM1/ARP response to MeJa lacks functional data. In this study, MeJa exposure induced DRM1/ARP expression for cluster 3, while in *A. thaliana*, the genes AtDRM1 and AtDRM11 are repressed after the MeJa treatment (Rae *et al.*, 2014). In rice, genes upregulated in dormant buds are also induced by JA. This implied a correlation between strigolactone-mediated axillary bud dormancy and JA (Luo *et al.*, 2019). JA is also a hormone with a key role in the regulation of the defense signaling (Pieterse *et al.*, 2011) and the responsiveness of the cluster 3 might indicate a possible role of this group in response defense. DRM1 and 2 yet were related to biotic or abiotic stress response (Rae *et al.*, 2014).

The time it takes for DRM1/ARP to change its expression after exposure to auxin suggests that those proteins can be classified as late response proteins to auxin. While genes for primary response are quickly altered after hormone exposure (commonly 5- 60 minutes), secondary response genes take more time (Abel and

Theologis, 1996). ARF is an example of a primary response gene to auxin (Shen *et al.*, 2015). Hence, our data suggest that the DRM1/ARP family is downstream ARF during the auxin response pathway. Other species show late induction of DRM1/ARP proteins after auxin exposure, including *Elaeagnus umbellata* and *Pyrus pyrifolia* (Kim *et al.*, 2007; Shi *et al.*, 2013). Interestingly, the auxin signaling pathway leads to late expression of DRM1/ARP in soybean, which suggests that this family is a repressor of the auxin signaling pathway.

3.4. Expression analysis of DRM1/ARP family clusters in response to *M. javanica* infection

The expression patterns for all clusters were analyzed after infection with nematode *M. javanica* in roots of soybean cultivar Desafio (susceptible). Clusters 1 and 3 were induced 3 days after infection, which suggests that this family has a role in compatible interactions between soybean and nematodes (Figure 5). DRM1/ARP were also related to biotic stress responses. Differential expression of DRM1/ARP during infection stress was previously demonstrated. Two genes of the DRM1/ARP family were identified from a resistant cultivar (PI 595099) cDNA library synthesized after nematode infection in soybean (De Sá *et al.*, 2012). DRM1/ARP genes were induced in a resistant peanut cultivar (*Arachis stenosperma*). A susceptible peanut cultivar showed reduced expression levels of DRM1/ARP after infection with *M. arenaria* (Proite *et al.*, 2007; Guimarães *et al.*, 2010). In *Nicotiana*, plants silenced for NbDRM3 were compromised in their pathogen-associated molecular pattern-triggered immunity (PTI) (Chakravarthy *et al.*, 2010). These data provide increasing evidence that DRM1/ARPs are involved in responding to biotic stress.

4. Conclusion

DRM1/ARP family is a protein family exclusive from plants and is highly conserved among plant species. Soybean presented the largest DRM1/ARP family among the species investigated, with 27 putative members. The time auxin takes to change the DRM1/ARP expression suggests that these genes can be classified as a late response to auxin, which indicates that this family is a repressor of the auxin signaling pathway in soybean. Finally, the data provided in this study show that, in soybean,

nematode infection increases DRM1/ARP expression. These data suggest that this soybean gene family may be an important link between the response to pathogens and plant growth and development. This is an increasing evidence that DRM1/ARPs are involved in responding to abiotic or biotic stresses, as well as the regulation of dormancy.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Figure Captions

Figure 1: Analysis by the K-means method and phylogenetic analysis of DRM1/ARP protein family members. A) DRM1/ARP protein cluster analysis. The red, green and blue colors refer to clusters 1, 2 and 3, respectively, of DRM1/ARP proteins in *G.max*, *M. truncatula*, *P. vulgaris* and *T. pratense*, *B. rapa* and *A. thaliana*. The cluster identification in the plot was performed by the K-means method (Steinley, 2006). B, C and D, phylogenetic analysis of DRM1/ARP proteins of the *G. max*, *M. truncatula*, *P. vulgaris*, *T. pratense*, *B. rapa* and *A. thaliana* of each protein cluster. Protein sequences were used to create the phylogenetic tree on MEGA7.0 by the neighbor-joining method, with bootstrap of 1000. The identification code of each protein is in supplementary table 2. B) Cluster 1 proteins. C) Cluster 2 proteins. D) Cluster 3 proteins.

Figure 2: Expression profile of clusters 1, 2 and 3 of DRM1/ARP Family in organs of soybean cultivar CD206. Relative quantification was measured by qPCR in four soybean organs. The asterisks indicate significant difference between the means of the organs analyzed by Bonferroni's Multiple Comparison test, with $P < 0.05$. The values refer to the means of three biological replicates (each replicate represents a pool of three different plants; and 20 different units were used for the seeds) and two technical replicates of each biological replicate. The expressions of *CYP2*, *ELF1A* and *UBC4* genes were used to normalize each sample.

Figure 3: Expression profile of clusters 1, 2 and 3 of DRM1/ARP Family and AUX/IAA gene in soybean cultivar CD206, in response to treatment with 100 μ M of IAA. The relative quantification was measured by qPCR at 1h, 6h and 12h after the IAA treatment. The asterisks indicate the significant difference between the treatment means (times 1h, 6h and 12h) and the control (time zero) by the Dunnett's Multiple Comparison test, with $P < 0.05$. The values represent the means of three biological replicates (each replicate represents a pool of three different plants) and two technical replicates of each biological replicate. The expressions of *CYP2*, *ELF1A* and *UBC4* genes were used to normalize each sample.

Figure 4: Expression profile of clusters 1, 2 and 3 of DRM1/ARP Family and VSP2 gene in soybean cultivar CD206, in response to treatment with 100 μ M of MeJA. The relative quantification was measured by qPCR at 1h, 6h and 12h after MeJA treatment. The asterisks indicate the significant difference between the treatment means (times 1h, 6h and 12h) and the control (time zero), by Dunnett's Multiple Comparison test with $P < 0.05$. The values correspond to the means of three biological replicates (each replicate represents a pool of three different plants) and two technical replicates of each biological replicate. The expressions of *CYP2*, *ELF1A* and *UBC4* genes were used to normalize each sample.

Figure 5: Expression profile of clusters 1, 2 and 3 of DRM/ARP Family in the susceptible soybean cultivar “Desafio”, during *M. javanica* infection. The relative quantification was measured by qPCR three days after inoculation. The asterisks indicate the significant difference between control (Mock) and treatment (Infected) by the T test, with $P < 0.05$. The values correspond to the means of three biological replicates and two technical replicates of each biological replicate. *TUA5* and *CYP2* expressions were used to normalize each sample.