Genome-Wide Identification and Expression Analysis of the Aux/IAA and Auxin Response Factor Gene Family in Medicago truncatula

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Abstract: Aux/IAA and auxin response transcription factor (ARF) genes are key regulators of auxin responses in plants. A total of 25 MtIAA and 40 MtARF genes were identified based on the latest updated Medicago truncatula reference genome sequence. They were clustered into 10 and 8 major groups, respectively. The homologs among M. truncatula, soybean, and Arabidopsis thaliana shared close relationships based on phylogenetic analysis. Gene structure analysis revealed that MtIAA and MtARF genes contained one to four concern motifs and they are localized to eight chromosomes, except chromosome 6 without MtARFs. In addition, some MtIAA and MtARF genes were expressed in all tissues, while others were specifically expressed in specific tissues. Analysis of cis-acting elements in promoter region and expression profiles revealed the potential response of MtIAA and MtARF genes to hormones and abiotic stresses. The prediction protein–protein interaction network showed that some ARF proteins could interact with multiple Aux/IAA proteins, and the reverse is also true. The investigation provides valuable, basic information for further studies on the biological functions of MtIAA and MtARF genes in the regulation of auxin-related pathways in M. truncatula.

Keywords: abiotic stress; Aux/IAA; auxin response factor (ARF); cis-acting elements; Medicago truncatula

1. Introduction

Indole-3-acetic (IAA) is the primary auxin in higher plants and regulates plant growth and development as well as responses to environmental stimuli [1,2]. The changes in auxin levels trigger downstream gene reprogramming through auxin response genes, such as the auxin/indole-3-acetic acid (Aux/IAA) family, the auxin response factor (ARF) family, and small auxin upregulated RNA (SAUR), and the auxin-responsive gretchen hagen 3 (GH3) family [3]. Aux/IAAs and ARFs are essential for auxin-mediated transcriptional regulation [4,5]. Aux/IAA proteins bind with ARFs for repressing activation of downstream auxin-responsive genes in the absence of auxin. Aux/IAA is ubiquitinated by interacting with TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB) receptors and subsequently degraded via the 26S proteasome under high concentrations of auxin [4,5]; ARFs are released for regulation of the expression of auxin-responsive genes [4,5].

Twenty-nine Aux/IAA gene family members are found in Arabidopsis [6]. Four highly conserved domains exist in most Aux/IAA proteins. Domain I has a conserved leucine repeat (LXXLXLX) motif that interacts with TOPLESS (TPL) protein (a co-repressor protein) to mediate auxin-dependent transcriptional repression [7]. Domain II is the auxin degro
with a conserved “GWPPV” motif that directly interacts with SCF^{TIR1} (SKP1/Cullin/F-box protein complex containing the transport inhibitor response 1 protein) and is associated with the turnover of Aux/IAA proteins [8]. A carboxy-terminal PB1 (Phox and Bem 1) domain is contained within a region that was previously called domain III/IV and functions to interact with ARF. It is, thus, involved in the regulation of ARF activity [9]. Aux/IAA is involved in the regulation of diverse cellular and developmental processes, including embryogenesis, axis formation and patterning, lateral root initiation, leaf expansion, vascular elongation, tropism, inflorescence and fruit development, apical dominance, and defense responses against pathogens [10–12]. For example, loss in the function of $iaa3/shy2$ mutation affects auxin homeostasis and the formation of lateral roots [13]. TIR1/AFB2 form specific sensing complexes with AtIAA6, AtIAA9 and/or AtIAA17 to modulate JA homeostasis and adventitious root initiation in the presence of auxin [14]. AtIAA33 maintains root distal stem cell identity and negatively regulates auxin signaling by interacting with AtARF10 and AtARF16 [15]. Aux/IAA family members are also identified in other plants, such as tomato (Solanum lycopersicon), cucumber (Cucumis sativus), maize (Zea mays), and rice (Oryza sativa) [16–19].

Twenty-three ARF members are identified in Arabidopsis [20]. Most ARF proteins are consisted of an N-terminal DNA-binding domain (DBD), a middle region (MR) and a C-terminal dimerization domain (CTD) [3]. The DBD belongs to B3-like family and enables ARFs to specifically bind with the TGTCTC auxin response elements (AuxREs) present in the promoters of various auxin-responsive genes [21]. The MR, whose sequence is less conserved, depends on ARF as a transcriptional activator or repressor depending on its amino acid composition [21,22]. The CTD domain is involved in homo- and hetero-interaction and inhibits its binding to the auxin-responsive elements under low auxin concentration conditions [23]. Some ARF genes conferring diverse biological processes have been functionally characterized [10]. For instance, AtARF7 and AtARF19 proteins are essential for auxin-mediated plant development by regulating both unique and partially overlapping sets of target genes [20]. AtARF2-4 and AtARF5 are essential for female and male gametophyte development [24]. AtARF8 regulates stamen elongation and endothecium lignification [25], while AtARF3 plays a distinct role during early flower development [26]. Some ARF family members are also identified in other plants, such as soybean (Glycine max), maize, potato, and Brachypodium distachyon [27–30].

Medicago truncatula is a model legume due to its small genome, self-pollination and many seeds, high genetic transformation efficiency, and large number of mutants [31]. Genome-wide analysis of Aux/IAA and ARF revealed that there are 17 Aux/IAA and 24 ARF genes in M.truncatula based on version M3.5 of the M. truncatula genome database [32,33]. Most of the MtIAA and MtARF genes were expressed in response to the early phase of S. meliloti infection, revealing the distinctive expression and function features of Aux/IAA and ARF family genes in Medicago truncatula during nodule formation and symbiotic interaction, respectively [32,33]. However, 25 Aux/IAA and 40 ARF members were isolated when we searched in version M4.0v1 of the M. truncatula genome database; the effect of abiotic stress on MtIAA or MtARF and the prediction of the interaction between MtIAA and MtARF have not been reported. Given the important role of Aux/IAA and ARF in regulation on plant growth and development, it is worth updating the information. The objectives of this study were to analyze the gene structures, chromosomal locations, phylogenetic relationships, motif organization, cis-acting element and expression patterns under salt, drought and cold treatments, and predicted protein interaction network of Aux/IAA and ARF genes in M. truncatula. The results provide a comprehensive understanding of the Auxin/IAA and ARF gene families in M. truncatula.
2. Results

2.1. Identification of IAA and ARF Members in M. truncatula

The IAA and ARF genes of Arabidopsis thaliana and soybean were used as query sequences to search for IAA and ARF genes in the genomes of M. truncatula. Twenty-five MtIAA and 40 MtARF genes were obtained, and their deduced peptides were confirmed after domain analysis, using Pfam and SMART databases. A total of 25 and 40 members were finally identified, and they were named MtIAA1 to MtIAA25 (Table 1) and MtARF1 to MtARF40 (Table 2), respectively, based on their locations on the chromosomes. The amino acid sequence of MtIAAs and MtARFs were further analyzed. The amino acids in length were ranged from 161 amino acids in MtIAA22 to 356 amino acids in MtIAA24, with an average of 237 in MtIAAs, while the average amino acid length of MtARFs was 642, ranging from 163 amino acids in MtARF29 to 1265 amino acids in MtARF1. The predicted molecular weight (MW) varied from 18.19 KDa to 38.67 KDa, and the theoretical isoelectric point (pI) varied from 4.77 to 9.00 in MtIAAs (Table 1), while the predicted MW varied from 18.06 KDa to 141.1 KDa, and pI varied from 5.14 to 8.87 in MtARFs (Table 2). The predicted grand average of hydropathicity (GRAVY) of all MtIAAs and MtARFs were negative, indicating that MtIAAs and MtARFs are hydrophilic proteins (Tables 1 and 2).

Table 1. Information on MtIAA proteins.

| Name       | Locus ID     | ORF (bp) | A.A. | MW (KDa) | pI     | GRAVY  |
|------------|--------------|----------|------|----------|--------|--------|
| MtIAA1     | Medtr1g040675| 561      | 186  | 20.76    | 6.75   | −0.554 |
| MtIAA2     | Medtr1g069495| 819      | 272  | 30.11    | 7.15   | −0.814 |
| MtIAA3     | Medtr1g070520| 738      | 245  | 27.48    | 7.57   | −0.689 |
| MtIAA4     | Medtr1g070830| 615      | 204  | 22.91    | 6.20   | −0.732 |
| MtIAA5     | Medtr1g080860| 762      | 253  | 27.51    | 6.75   | −0.512 |
| MtIAA6     | Medtr1g085750| 531      | 176  | 19.80    | 5.56   | −0.535 |
| MtIAA7     | Medtr1g093240| 711      | 236  | 25.72    | 8.60   | −0.552 |
| MtIAA8     | Medtr1g093350| 537      | 178  | 19.98    | 7.68   | −0.752 |
| MtIAA9     | Medtr1g109510| 537      | 178  | 20.19    | 4.77   | −0.493 |
| MtIAA10    | Medtr2g100780| 810      | 269  | 29.96    | 8.86   | −0.863 |
| MtIAA11    | Medtr2g101500| 981      | 326  | 35.36    | 8.11   | −0.539 |
| MtIAA12    | Medtr2g102490| 597      | 198  | 22.50    | 8.80   | −0.529 |
| MtIAA13    | Medtr3g106850| 642      | 213  | 24.73    | 9.00   | −0.883 |
| MtIAA14    | Medtr4g011880| 537      | 178  | 20.14    | 6.21   | −0.598 |
| MtIAA15    | Medtr4g115075| 627      | 208  | 24.00    | 6.16   | −0.853 |
| MtIAA16    | Medtr4g124300| 555      | 184  | 20.88    | 6.51   | −0.497 |
| MtIAA17    | Medtr4g128070| 522      | 173  | 19.22    | 7.78   | −0.524 |
| MtIAA18    | Medtr5g030710| 1008     | 335  | 36.26    | 8.52   | −0.468 |
| MtIAA19    | Medtr5g067350| 1044     | 347  | 38.01    | 8.62   | −0.567 |
| MtIAA20    | Medtr6g488150| 765      | 254  | 28.32    | 8.43   | −0.580 |
| MtIAA21    | Medtr7g096090| 753      | 250  | 27.90    | 5.36   | −0.558 |
| MtIAA22    | Medtr7g110790| 486      | 161  | 18.19    | 5.68   | −0.358 |
| MtIAA23    | Medtr8g014520| 816      | 271  | 30.12    | 8.28   | −0.755 |
| MtIAA24    | Medtr8g067530| 1071     | 356  | 38.67    | 6.38   | −0.455 |
| MtIAA25    | Medtr8g103030| 882      | 293  | 31.89    | 8.04   | −0.509 |

Table 2. Information on MtARF proteins.

| Name      | Locus ID     | ORF (bp) | A.A. | MW (KDa) | pI     | GRAVY  |
|-----------|--------------|----------|------|----------|--------|--------|
| MtARF1    | Medtr1g024025| 3798     | 1265 | 141.16   | 5.26   | −0.439 |
| MtARF2    | Medtr1g058210| 1380     | 459  | 51.30    | 8.32   | −0.383 |
| MtARF3    | Medtr1g064430| 2070     | 689  | 76.95    | 6.69   | −0.475 |
three species, indicating that the Aux/IAA differentiation time was earlier than the species in subdivided into four subgroups (I to IV), containing 57 members (12 in listed in Table S1. Referring to those in evolutionary relationship (Figure 1A,B). The sequences of MtIAA and MtARF proteins are (63, 55) were aligned to generate unrooted phylogenetic trees for evaluation of their evo-

2.2. Phylogenetic Analysis of IAAs and ARFs among M. truncatula, Soybean and Arabidopsis

All IAA and ARF proteins in M. truncatula (25, 40), Arabidopsis (29, 23) and soybean (63, 55) were aligned to generate unrooted phylogenetic trees for evaluation of their evolutional relationship (Figure 1A,B). The sequences of MtIAA and MtARF proteins are listed in Table S1. Referring to those in Arabidopsis thaliana, soybean, and Brassica napus (20,34,35), the Aux/IAA family was classified into two groups, A and B. Group A was subdivided into four subgroups (I to IV), containing 57 members (12 in M. truncatula, 13 in Arabidopsis, and 32 in soybean) (Figure 1A), while the group B was subdivided into six subgroups (V to X), containing 60 members (13 in M. truncatula, 16 in Arabidopsis, and 31 in soybean) (Figure 1B). Each subgroup contained the Aux/IAA members of the above three species, indicating that the Aux/IAA differentiation time was earlier than the species.
differentiation. In addition, the phylogenetic tree showed that Aux/IAA members in *M. truncatula* were closely related to those in soybean.

ARFs were clustered into eight groups (I to VIII). According to the amino acid sequence of MR in the middle region of MtARF, MtARF members could be divided into transcriptional activators and repressors. MtARF1, -9, -11, -14, -20, -29, -30, -37 and -39 were predicted to be transcription activators containing a Q-, S-, and L-rich MR domain in subclasses III to V, while MtARF5, -16, -33, -36, -38 and -40 containing a S-, P-, L-, and G-rich MR domain were predicted to be transcription repressors in subclass I. The MtARF members from subclasses I, II and IV–VII were more closely related to those in soybean and *Arabidopsis* than those in subclass VIII, indicating a trend in the development of ARF family members across different plant species.

2.3. Gene Structure and Domain Architecture

The arrangement of exons/introns was used for analysis of the gene structure of MtIAAs and MtARFs. One to six introns were found in the Aux/IAA members. Most of the MtIAAs had multiple introns. One intron was found in two MtIAAs (MtIAA8, MtIAA17); two introns in two MtIAAs (MtIAA4, MtIAA14); three introns in eight MtIAAs; four introns in ten MtIAAs; five in MtIAA18 and MtIAA19; and six in MtIAA24 (Figure 2A). Except for one member (MtARF28) without an intron, MtARFs had 1 to 21 introns. One intron was found in eleven MtARFs; two introns in six MtARFs (MtARF3, MtARF25, MtARF27, MtARF32, MtARF34 and MtARF35); three in MtARF17 and MtARF29; five in MtARF13 and MtARF24; nine in MtARF8, MtARF19 and MtARF40; eleven in MtARF12 and MtARF18; twelve in MtARF11, MtARF20, MtARF33 and MtARF38; thirteen in MtARF5, MtARF9, MtARF14, MtARF16, MtARF30, MtARF36, MtARF37 and MtARF39; and twenty-one introns in MtARF1 (Figure 2D).
Figure 2. Characterization of MtIAA genes and MtARF proteins. (A,D) exon–intron structure distribution; (B,C) protein motif.
A typical Aux/IAA gene contains four structure motifs (I, II, III and IV) \[3,36\]. Most MtIAA members (17) contained four motifs. Two members (MtIAA13 and MtIAA15) contained three motifs, missing motif I, while five MtIAA members (MtIAA6, MtIAA9, MtIAA16, MtIAA17 and MtIAA22) contained only two motifs, missing motifs I and II (Figure 2B). A total of 10 conserved motifs in the MtARFs were identified. In fact, the B3 domain corresponded to motifs 2, 3 and 4; the ARF domain consisted of motifs 6, 8 and 9; and motifs 7 and 10 formed the CTD domain. In addition, B3 domain and the ARF domain constituted a conserved DBD structure. There were 15 ARF members (MtARF1, 5, 9, 11, 12, 14, 16, 18, 20, 30, 33, 36, 37, 38 and 39), which contained all of the three domains. Except MtARF29, which only had the B3 domain, the rest of the 24 MtARFs lacked the CTD domain (Figure 2C).

2.4. Chromosomal Location and Synteny Analysis of MtIAA and MtARF Genes

The MtIAA and MtARF gene locations were mapped on chromosomes. All chromosomes had MtIAA and MtARF genes, except for chromosome 6, lacking MtARF. The largest number of MtIAAs (9) and MtARFs (12) were located on chromosomes 1 and 5, respectively, while the fewest of them were on chromosomes 6 and 3 (Supplementary Figure S1).

Collinearity diagrams among MtIAAs and MtARFs were further analyzed. The results showed that some MtIAA and MtARF genes underwent gene duplication in the genomes of the M. truncatula genome; for example, MtIAA3/MtIAA21, MtIAA6/MtIAA22, MtARF8/MtARF19, MtARF11/MtARF20 and MtARF12/MtARF18 were pairs of segmental duplicates, respectively (Figure 3). Six pairs of homologous IAA and three pairs of homologous ARFs were identified between M. truncatula and A. thaliana, respectively, but fifty-two pairs of orthologous IAA and fifty-four pairs of orthologous ARFs were identified between M. truncatula and soybean, respectively (Figure 3 and Table S2). Six MtIAAs (MtIAA5, MtIAA6, MtIAA13, MtIAA16, MtIAA17 and MtIAA22) and three MtARFs (MtARF15, MtARF18 and MtARF36) had one homologous gene in A. thaliana, respectively, while four MtIAAs (MtIAA2, MtIAA6, MtIAA11 and MtIAA22) and five MtARFs (MtARF8, MtARF11, MtARF19, MtARF20 and MtARF38) had four homologous genes in soybean. In addition, four MtIAAs (MtIAA3, MtIAA4, MtIAA21 and MtIAA23) and five MtARFs (MtARF15, MtARF17, MtARF30, MtARF34 and MtARF36) had three homologous genes; the others had two homologous genes in soybean. The percentage of identity between pairs of paralogous MtIAA and MtARF proteins ranged from 65.54% to 71.04% and 65.52% to 78.91% in M. truncatula, respectively (Table S2). The identity of IAA and ARFs between pairs of orthologous ranged 33.06% to 77.59% and 48.84% to 59.87% between M. truncatula and A. thaliana, respectively, while the identity of IAA and ARFs between pairs of orthologous ranged from 43.93% to 80.75% and 38.40% to 90.91%, respectively, between M. truncatula and soybean. The high percentages of identity in IAA and ARFs between M. truncatula and soybean suggest that MtIAA and MtARF protein sequences and functions were highly conserved and that M. truncatula is closely related to soybean.
2.5. Spatial and Temporal Expression of MtIAAs and MtARFs

The spatial and temporal expression of MtIAAs and MtARFs were analyzed based on the microarray data (MtGEA, https://mtgea.Noble.orgv3/ (accessed on 24 January 2021)). The data of 18 MtIAAs and 24 MtARFs can be found in the dataset (Table S3). MtIAA2, MtIAA7, MtIAA8, MtIAA11, MtIAA18 and MtIAA24 were highly expressed in all tissues (roots, stems, leaves, flowers, petioles, pods and seeds) (Figure 4A and Table S3), indicating that they may have diverse functions. MtIAA1, MtIAA3 and MtIAA4 were mainly expressed in roots, stems, leaves, petioles and flowers. MtIAA23 was preferentially expressed in flowers, while MtIAA19 and MtIAA25 were expressed in roots, petioles, stems, flowers, pods and seeds, but not in leaves.

Figure 4. Spatial and temporal expression of MtIAA and MtARF genes. (A) MtIAA; (B) MtARF. MtIAA and MtARF expression levels are shown as the log2-based fluorescence intensity values from MtGEA (https://mtgea.Noble.orgv3/ (accessed on 24 January 2021)). DAP indicates days after pollination.
MtARF5, MtARF9, MtARF11, MtARF16, MtARF20, MtARF37, MtARF38 and MtARF39 showed relatively high expression in all tissues (roots, stems, leaves, flowers, petioles, pods and seeds); among them, MtARF38 had the highest expression (Figure 4B), indicating that MtARF38 may play an important role in the regulation of growth and development in M. truncatula. MtARF1 and MtARF14 were mainly expressed in roots, stems, flowers, petioles, pods and seeds, but not in leaves. MtARF18 was mostly expressed in roots, stems, leaves, flowers, petioles and pods, but not in seeds, while MtARF40 was only expressed in seeds. MtARF33 was majorly expressed in seeds and roots (Figure 4B and Table S3).

2.6. Analysis of cis-Acting Element in the Promoter Region of MtIAA and MtARF Genes

To understand the potential regulation of MtIAA and MtARF expression, a 2 kb promoter sequence of MtIAAs and MtARFs was analyzed; the results are listed in Table S4. Light (Box4), anaerobic (ARE), MeJA (CGTCA-motif), gibberellin (GARE-motif) and ABA (ABRE) response elements were abundant in the promoter of MtIAAs and MtARFs, while salicylic acid (TCA-motif), drought (MBS), auxin (AuxRR-core), cold (LTR), endosperm, meristem and circadian response elements were also found in the promoters (Figure 5A,B). Ten MtIAAs and twenty-six MtARFs had a drought response element; seven MtIAAs and seventeen MtARFs had a cold response element; and five MtIAAs and twenty MtARFs had a defense and stress response element. Seven MtIAAs and sixteen MtARFs had an endosperm response element, while six MtIAAs and twelve MtARFs had a circadian response element (Figure 5A,B). The results indicated that MtIAAs and MtARFs may be responsive to plant hormones, growth and development as well as various abiotic stresses.
2.7. Responses of MtIAAs and MtARFs to Salt, Drought and Cold

The responses of MtIAAs and MtARFs’ expression to salt and drought stress were obtained from MtGEA (https://mtgea.Noble.orgv3/(accessed on 24 January 2021)). MtIAAs and MtARFs’ expression patterns were altered after salt and drought stress (Table S5). MtIAA23 expression was upregulated after 6 h of salt treatment by placing on a 1/2 MS medium containing 180 mM NaCl. MtIAA14 was downregulated with the extension of time, and MtIAA5 was downregulated after 48 h of salt stress (Figure 6A). MtARF14 was downregulated after 6 h, while MtARF5, MtARF9, MtARF20, MtARF37 and MtARF39 showed the highest expression after 14 h of salt stress (Figure 6B). In the hydroponic experiment, by treatment in a nutrient solution containing 200 mM NaCl, MtIAA4 was upregulated within 5 h and downregulated at 10 h after treatment, whereas MtIAA14 was downregulated continuously in response to the treatment (Figure 6C). Most of MtARFs’ transcripts were invariable in the hydroponic treatment experiment, except for MtARF33 and MtARF14, whose expression was decreased significantly (Figure 6D).
Figure 6. Responses of MtIAAs and MtARFs' expression to salt and drought stress. The microarray data were retrieved from M. truncatula Gene Expression Atlas (MtGEA, https://mtgea.Noble.orgv3/ (accessed on 24 January 2021)). (A, B) Two-day-old seedlings were treated by placing in 1/2 MS medium containing 180 mM NaCl for 0, 6, 24 and 48 h for salt stress. (C, D) Two-week-old seedlings were placed in a nutrient solution containing 200 mM NaCl for 1, 2, 5, 10 and 24 h as hydroponic treatment, with those growing in the nutrient solution as the control. (E–H) The 24-day-old seedlings growing in soil underwent withheld irrigation for 14 d of drought treatment before rewatering. MtIAAs and MtARFs expression levels are indicated as the log2-based fluorescence intensity values.

Compared to the induced expression of MtIAA13, MtIAA23 and MtARF3, most of the MtIAA and MtARF transcripts were unaltered in shoots during drought treatment (Figure 6E, F). On the other hand, the MtIAA14 and MtARF4 transcripts in the roots were decreased after drought treatment followed by an increase after rewatering (Figure 6G, H), whereas MtARF3 and MtARF20 were induced by drought treatment followed by a decrease after rewatering (Figure 6H). The results indicated that some of MtIAA and MtARF genes may participate in salt and drought responses.

Six MtIAAs and six MtARFs that have LTR cis-acting element in the promoter regions were selected for analysis of gene expression in response to cold (Table S6). The MtIAA1, MtIAA14 and MtARF5 transcripts were significantly reduced after 2 h of cold treatment but showed no significant difference after 12 h. MtIAA5, MtIAA7, MtIAA21, MtIAA24, MtARF1, MtARF18, MtARF20, MtARF37 and MtARF38 transcript levels were significantly reduced at 2 and 12 h after cold treatment (Figure 7). The results suggest that MtIAAs and MtARFs, which have a LTR cis-acting element in the promoter regions, may participate in cold adaptation in M. truncatula.
Figure 7. Relative expression of MtIAA1 (A), MtIAA5 (B), MtIAA7 (C), MtIAA14 (D), MtIAA21 (E), MtIAA24 (F), MtARF1 (G), MtARF5 (H), MtARF18 (I), MtARF20 (J), MtARF37 (K) and MtARF38 (L) in response to cold. The presented are means and standard errors of three independent experiments. The different letters in a column denote significant differences among the treatments at $p < 0.05$.

2.8. Predicted MtIAA and MtARF Family Interaction Networks

A protein–protein interaction network between MtIAAs and MtARFs was predicted by the STRING (https://www.string-db.org/(accessed on 20 March 2021)) software. 18 MtIAAs and 24 MtARFs were found to form a protein–protein interaction network (Figure 8). The results showed that some MtARF proteins could interact with multiple MtIAAs, while some MtIAAs could interact with multiple MtARF. It is notable that four MtARFs (MtARF5, MtARF19, MtARF36 and MtARF39) that function as activators may interact strongly with most of MtIAA proteins. In addition, MtARF29 may interact with MtIAA12 and MtIAA21 as well as multiple MtARFs. Moreover, a lot of MtARF genes showed co-expression correlation, indicating that these genes might be involved in the same regulatory pathway. For example, MtARF5 had high co-expression levels with 11 MtIAAs and 3 MtARFs, and MtARF19 with 10 MtIAAs and 2 MtARFs, indicating that MtARF5 or MtARF19 might be a key regulator among the 40 MtARFs (Figures 8 and S2).
3. Discussion

Auxin plays a critical role in controlling plant growth and developmental and physiological processes, while IAA and ARF are key components in auxin signaling for regulating downstream reactions [4]. The numbers of IAA and ARF members are different among plant species; for example, there are 29 IAA and 23 ARF in Arabidopsis [6,20], 31 IAA and 25 ARF in rice [19,22], and 63 IAA and 55 ARF in soybean [27,34]. A total of 17 IAA and 24 ARF genes were reported in M. truncatula [32,33], while a total of 25 MtIAA and 40 MtARF genes were identified in this study based on the updated genome data. MtIAAs and MtARFs showed extensive variations in ORF length, predicted MW and pI, which was also observed in rice IAA [19] and Brachypodium distachyon ARF [30]. The variations implied that the diverse MtIAA and MtARF proteins might function under different microenvironments. All MtIAAs and MtARFs had negative GRAVY, suggesting that they are hydrophilic proteins. Like those in other plant species [28,29,37], most MtIAA and MtARF genes have multiple introns.

A typical Aux/IAA protein contains four conserved domains designated as I, II, III and IV [3,35]. A total of 17 MtAux/IAA proteins contained 4 domains, while the others lost at least 1 domain (Figure 2B). MtIAA13 and MtIAA15 proteins lost domain I, indicating that they might experience a loss in capacity in recruiting TPL co-repressors and thus, lost the function as a repressor in auxin signaling. In addition, MtIAA12 protein, like AtIAA20 [4], lost domain II, indicating that it should not be degraded under increased levels of auxin [4]. Recent studies have shown that, instead of degrading non-canonical Aux/IAA proteins by TIR1/AFB, auxin stabilizes non-canonical Aux/IAA proteins by
phosphorylation of upstream protein kinases; for instance, auxin regulates the stability of non-canonical AtIAA32 and AtIAA34 proteins through transmembrane kinases (TMK), and then regulates gene expression through ARF transcription factors to mediate the differential growth during apical-hook development [38]; meanwhile, auxin also regulates the stability of non-canonical AtIAA33 protein through MITOGEN-ACTIVATED PROTEIN KINASE 14 (MPK14) and does not affect AtIAA33 gene expression [15]. Another explanation is that the Aux/IAA proteins are too low in the tissues to be able to affect plant growth and development [35], even some of the deduced sequences might be pseudogenes, because no information about expression is available for several of them [36]. The expression level of MtIAA6, MtIAA17 and MtIAA22 genes lacking domain II was very low in all major tissues, compared with the canonical Aux/IAA genes, while the others (MtIAA9, 12 and 16) showed no available information (Figure 4A), which was consistent with those in Brassica napus lacking domain II [35]. The others MtIAAs (MtIAA6, MtIAA9, MtIAA16, MtIAA17 and MtIAA22) lost both domains I and II, indicating that they could neither be a repressor nor be rapidly degraded in auxin signaling. The truncated Aux/IAA proteins also exist in multiple plant species. Domains I and II are lost in AtIAA29 and AtIAA33 in Arabidopsis, and OsIAA4, OsIAA27, OsIAA28 and OsIAA29 in rice [6,19]; Domain II is lost in CaIAA5, -11, -12, -16, -17 and -19 in chickepa; and GmIAA5, -6, -13, -23, -31, -35, -37, -39, -40, -42, -53 and -60 in soybean [34]. Domain III or IV is lost in PeAA1, -18, and -24 proteins in moso bamboo [39]. Thus, the variations in these domains are associated with diverse functions of Aux/IAA in the auxin signaling pathway.

A typical ARF protein contains three domains, designated as DBD, MR and CTD [3]. ARF proteins rely on the DBD to bind specifically to auxin response elements (AuxRE: TGTCTC) in the promoters of auxin responsive genes [9]. The amino acid composition of the MR region depends on it as an activator or repressor [33]. The CTD is involved in homo- and hetero-interactions among ARFs [23]. A total of 14 MtARF proteins have complete domains, while MtARF29 lack MR and CTD domains and the others lack the CTD domain (Figure 2C). Eight MtARFs (MtARF1, -9, -11, -14, -20, -30, -37 and -39) are predicted to be transcriptional activators based on the fact that glutamine (Q), serine (S) and leucine (L) are enriched in the MR domain, while seven (MtARF5, -12, -16, -18, -33, -36 and -38) are putative transcriptional repressors because S, L, proline (P) and glycine (G) are enriched in the MR region. A total of 24 MtARF proteins, rich in S, L, proline (P) and glycine (G) in the MR region, are putative transcriptional repressors that lack a CTD. This means that the ratio of activator/repressor numbers of MtARFs is 0.26, which is consistent with previous reports [33]. Our investigation provides insight into understanding the potential role of MtARF genes in the regulation of plant developmental processes and responses to environmental stresses. Canonical auxin responsive transcription factor ARF family proteins bind to the promoter region of Aux/IAA genes through their CTD domain and are regulated by TIR1/AFB receptors [3]. The loss of CTD in MtARFs revealed that they may function in an auxin-independent manner. AtARF3 that lacks the CTD domain did not bind with the elements of the canonical TIR1/AFB signaling pathway and functions, independent of the TIR1/AFB receptor [40].

TBtools (v1.09854, Chengjie Chen, Guangzhou, China) software was used for analyzing the synteny of IAA or ARF genes among M. truncatula, A. thaliana and soybean. A total of 6 MtIAA-AtIAA pairs but 52 MtIAA-GmIAA pairs were observed among Aux/IAA family, and 3 MtARF-AtARF pairs but 54 MtARF-GmARF pairs among the ARF family. The results support that M. truncatula is phylogenetically closer with soybean than with Arabidopsis. Two pairs of MtIAAs (MtIAA3/MtIAA21, MtIAA6/MtIAA22,) and three pairs of MtARFs (MtARF8/MtARF19, MtARF11/MtARF20 and MtARF12/MtARF18) belong to segmental duplication, indicating that M. truncatula has undergone local gene duplication and shares an ancient round of gene duplication with other legume species [31].

The spatial expression of genes is related to their potential functions. Some MtIAA and MtARF genes showed specific and overlapping expression patterns in various tissues and developmental stages, implying that they may have specific functions. Seven MtIAAs
involved in abiotic stress adaptation in MtIAAs treatment in regulates the expression of downstream auxin response genes and causes a series of auxin-related responses [3,23]. In addition, six responses to salt stresses. Four AtIAA7 and MtARF genes participated in hormonal or abiotic stresses when specific cis-elements were found in their promoter regions, such as MtARF38 and MtARFs (MtARF3, MtARF16, MtARF33 and MtARF38) were highly expressed in roots, suggesting that they are associated with flowering and root regulation, respectively. AtIAA7, the homolog of MtIAA7, is involved in the regulation of flowering time via the negatively regulating expression of GA20ox1 and GA20ox2 under short-day light conditions in Arabidopsis [41]. AtARF7, the homolog of MtARF20, is involved in the regulation of lateral root formation via activating LBD/ASL genes in Arabidopsis [42]. Some Aux/IAA and ARF proteins regulate gene transcription during leaf growth in the tomato, such as SIILAA1, SIILAA7, SIILAA19 and SIILAA24 [16], while 12 IAA genes were upregulated in leaves in Brassica napus [35]. Five PelIAAs (PelAA1, PelAA2, PelAA6, PelAA8 and PelAA16) and four PeARFs (PeARF8, PeARF14, PeARF18 and PeARF19) were highly expressed in shoots [38], and GmIAA45 and GmIAA51 transcripts were found in soybean shoots [34].

Some promoter elements (LTR, MBS, ABRE, CGTCA-motif, AuxRR-core, Box4, TCA-element and TATC-box) were enriched multiple times in the promoter regions of MtIAA and MtARF genes. Some IAA and ARF genes participated in hormonal or abiotic stresses responses when specific cis-elements were found in their promoter regions, such as AtIAA7 [41], SIILAA2, SIILAA11, SIILAA17, SIILAA19 and SIILAA29 [16], OsIAA20 [43], OsARF16 [24], SIARF2B, SIARF5 and SIARF9A [44], BdARF17 and BdARF23 [30]. Two MtARF genes (MtARF14 and MtARF18) and more than half of the MtIAA genes showed extensive responses to salt stresses. Four MtIAA genes (MtIAA2, MtIAA7, MtIAA14 and MtIAA23) and MtARF genes (MtARF3, MtARF11, MtARF14 and MtARF20) showed extensive responses to drought stresses, respectively. Similarly, many IAA and ARF genes in other plant species were induced by drought treatment [29,30,45,46]. Transcripts of BdARF5, BdARF12, SbIAA1, SbIAA26 and SbARF3 were up-regulated substantially following salt stress [30,46]. Six MtIAA genes showed downregulation in response to cold treatment. The downregulation of Aux/IAAs leads to the release of the inhibited ARF gene so that ARF regulates the expression of downstream auxin response genes and causes a series of auxin-related responses [3,23]. In addition, six MtARF genes were also downregulated after cold treatment. Twenty BdARFs and CalAA3 and CalAA7 transcripts were induced after cold treatment in Brachypodium distachyon and chickpea, respectively [30,34]. The responses of MtIAAs and MtARFs to drought, salt and cold suggest that MtIAAs and MtARFs are involved in abiotic stress adaptation in M. truncatula.

Protein-protein interactions are critically important to many processes, such as signal transduction and regulation of gene expression. Auxin responses are mediated by interaction between ARF and Aux/IAA proteins [7]. Therefore, it is significant to study the interaction between IAA and ARF in M. truncatula. In this study, the protein-protein interaction networks included 161 interaction combinations between 18 MtIAAs and 24 MtARFs (Figure 8). A total of 213 specific interactions between 19 ARFs and 29 Aux/IAA were identified in Arabidopsis, and up to 70% of ARF interacted with Aux/IAA factors by integrating co-expression maps with protein-protein interaction data [47]. Moreover, we observed that a single ARF protein could interact with multiple Aux/IAA and the reverse is also true; for example, MtARF29 interacted with MtIAA12 and MtIAA21 as well as a large number of MtARFs, which is consistent with the observation that SIARF2A interacts with five SIILAA, and SIARF6A interacts with at least 11 SIILAA in the tomato plant [48]. Similarly, transcriptional activators AtARF5, AtARF6, AtARF7, AtARF8 and AtARF19 interacted with almost all Aux/IAA proteins in Arabidopsis [47]. The predicted interaction networks and the co-expression network in this study provide clues for further investigations on the regulation of MtAux/IAA-MtARF on the growth, development and adaptation to environmental stresses in M. truncatula.
4. Material and Methods

4.1. Identification of IAA and ARF Genes in M. truncatula

The protein sequences of M. truncatula Mt4.0v1 were downloaded from Phytozome 12 (https://phytozome.jgi.doe.gov/pz/portal.html (accessed on 22 October 2020)). The reported IAA and ARF gene sequences in A. thaliana were downloaded from the TAIR database (https://www.arabidopsis.org/ (accessed on 22 October 2020)) and as queries for BLASTP search. The potential IAA and ARF genes in M. truncatula were searched via the NCBI database. Moreover, the protein sequences of GmIAAs and GmARFs were downloaded from SoyBase (https://www.soybase.org/ (accessed on 22 October 2020)) and used to search against the M. truncatula proteome. According to the main characteristics of the Aux/IAA protein family (Pfam:02309 AUX/IAA family) and ARF gene family (Pfam:02309: AUX/IAA family; Pfam 06507: Auxin_resp; Pfam 02362: B3 DNA binding domain) [32,33], IAA and ARF candidate genes were screened to distinguish the IAA and ARF homologous genes in M. truncatula. All generated non-redundant protein sequences were detected by SMART (http://smart.embl-heidelberg.de/ (accessed on 30 October 2020)) and InterProScan (http://www.ebi.ac.uk/interpro/ (accessed on 30 October 2020)) for the presence of major characteristic structures.

4.2. Analysis of Conserved Domain, Gene Structure and Characterization of MtIAA and MtARF Genes

Using the ProtParam tool of ExPASy (http://web.expasy.org/protparam/ (accessed on 28 October 2020)), we analyzed the physical and chemical characteristics, containing the molecular weight (MW), theoretical point (pI) and grand average of hydropathicity (GRAVY) of MtIAA and MtARF proteins. Using the TBtools (Toolbox for Biologists) program with default parameters, we analyzed the exon–intron structure of MtIAA and MtARF genes [49]. Using the MEME tool (http://meme-suite.org/tools/meme (accessed on 4 November 2020)), we analyzed the conserved motifs, with the minimum width of motifs as 10, the maximum width of motifs as 40 and the other parameters as default values.

4.3. Phylogenetic Relationships of MtIAA and MtARF Proteins in M. truncatula, Arabidopsis and Soybean

Using ClustalX with default parameters, we performed the multiple alignments for MtIAA and MtARF proteins, respectively. Using MEGA X with the maximum-likelihood (ML) method and 1000 bootstrap replicates, we analyzed the phylogenetics of IAAAs and ARFs in M. truncatula, Arabidopsis and soybean [50].

4.4. Chromosomal Locations of MtIAA and MtARF Genes

Using the sequence of MtIAA and MtARF genes, we searched their chromosomal locations in M. truncatula genome databases, such as Phytozome 12 (https://phytozome.jgi.doe.gov/pz/portal.html (accessed on 22 October 2020)). Using the TBtools software, we analyzed the chromosomal locations and homologous relationship of MtIAA and MtARF genes [49]. Using the Multiple Collinearity Scan toolkit (MCScanX), we analyzed the gene duplication events [49].

4.5. Analysis of cis-Acting Elements of MtIAA and MtARF Genes

Genomic DNA sequences of 2000 bp upstream of each MtIAA and MtARF transcription start site were obtained from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html (accessed on 22 October 2020)); using the PlantCARE database (http://bioinformatics. psb.ugent.be/webtools/plantcare/html/ (accessed on 28 January 2021)), we analyzed the cis-acting elements [51].
4.6. Analysis of Microarray Expression Profile

The genome-wide microarray data of *M. truncatula* in different tissues at various developmental stages and the response to drought and salt were searched from *M. truncatula* Gene Expression Atlas (MtGEA, https://mtgea.noble.org/v3/ (accessed on 24 January 2021)). Using the TBtools (v1.09854, Chengjie Chen, Guangzhou, China) software, we analyzed the transcript data and the normalized expression data of *MtIAAs* and *MtARFs* to generate heat [49].

4.7. Analysis of Relative Expression of MtIAAs and MtARFs in Response to Cold

The seeds of *M. truncatula* (R108) were treated with sandpaper to break the physical dormancy, and then placed on wet paper towel to absorb the water. After placing in a freezer at four °C for three days for vernalization treatment, the seeds were moved to room temperature for germination, followed by sowing in a plastic pot filled with soil. The plants were grown in a greenhouse for four weeks under natural light with temperature ranging from 20 to 28 °C, and then transferred to a growth chamber at 5 °C with a 12 h photoperiod under 200 μmol m⁻² s⁻² light for cold treatment, while those in a growth chamber at room temperature were used as the control. Leaves (0.1 g) were harvested for isolation of total RNA, using RNAprep pure Plant Kit (TIANGEN, Beijing, China), according to the manufacturer’s instructions. The cDNA synthesis and quantitative RT-PCR (qRT-PCR) were conducted as previously described [52]. Relative expression was calculated by $2^{-\Delta\Delta C_{t}}$. The *MtActin* gene was used as the internal control. The primers were listed in Table S8.

4.8. Predicted Protein Interaction Network and Co-Expression Network Construction

The interacting networks of MtIAA and MtARF proteins were integrated in the STRING (https://www.string-db.org/ (accessed on 20 March 2021)) software, and the co-expression network data were exported from STRING and calculated by Microsoft Excel 2019.

5. Conclusions

A total of 25 *MtIAA* and 40 *MtARF* genes were identified in *M. truncatula* based on version Mt4.0v1 of the *M. truncatula* genome database. Analysis of the intron-exon structure revealed that IAA and ARF gene families are evolutionarily conserved. Synteny analysis showed that tandem duplication probably participated in driving the *MtIAA* and *MtARF* genes’ evolution. *MtIAA* and *MtARF* genes were expressed in all organs detected, while some genes showed tissue-specific expression. The cis-acting elements responsive to plant hormones were enriched in the promoter of *MtIAAs* and *MtARFs*, while salicylic acid (TCA-motif), drought (MBS), cold (LTR), endosperm, meristem and circadian response elements were found in the promoter of some *MtIAAs* and *MtARFs*. Many *MtIAAs* and *MtARFs* were regulated by drought, salt and cold. The protein-protein interaction predicted 161 interaction combinations between 18 *MtIAAs* and 24 *MtARFs*. The study provides a valuable resource for further studies on the biological functions of MtIAA and MtARF genes in the regulatory mechanisms of auxin-related pathways in *M. truncatula*.

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