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The RNAome landscape of tomato during arbuscular mycorrhizal symbiosis reveals an evolving RNA layer symbiotic regulatory network

Zhen Zeng[1,2], Yang Liu[1,2], Xing-Yu Feng[1], Sai-Xi Li[1], Xing-Mei Jiang[1], Jian-Qun Chen[1,*] and Zhu-Qing Shao[1,*]

[1]State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210023, China

[2]These authors contributed equally.

***Correspondence: Jian-Qun Chen (chenjq@nju.edu.cn), Zhu-Qing Shao (zhuqingshao@nju.edu.cn)**

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ABSTRACT

Arbuscular mycorrhizal symbiosis (AMS) is an ancient plant-fungus relationship that is widely distributed in terrestrial plants. The formation of symbiotic structures and bidirectional nutrient exchange requires the regulation of numerous genes. However, the landscape of RNAome during plant AMS involving different types of regulatory RNA is poorly understood. In this study, a combinatorial strategy utilizing multiple sequencing approaches was used to decipher the landscape of RNAome in tomato, an emerging AMS model. The annotation of the tomato genome was improved by a multiple-platform sequencing strategy. A total of 3,174 protein-coding genes were upregulated during AMS, 42% of which were alternatively spliced. Comparative-transcriptome analysis revealed that genes from 24 orthogroups were consistently induced by AMS in eight phylogenetically distant angiosperms. Seven additional orthogroups were specifically induced by AMS in all surveyed dicot AMS host plants. However, these orthogroups were absent or not induced in monocots and/or non-AMS hosts, suggesting a continuously evolving AMS-responsive network in addition to a conserved core regulatory module. Additionally, we detected 587 lncRNAs, ten miRNAs, and 146 circRNAs that responded to AMS, which were incorporated to establish a tomato AMS-responsive, competing RNA-responsive endogenous RNA (ceRNA) network. Finally, a tomato symbiotic transcriptome database (TSTD, https://efg.nju.edu.cn/TSTD) was constructed to serve as a resource for deep deciphering of the AMS regulatory network. These results help elucidate the reconfiguration of the tomato RNAome during AMS and suggest a sophisticated and evolving RNA layer responsive network during AMS processes.

Key words: tomato, RNAome landscape, arbuscular mycorrhizal symbiosis, evolution, ceRNA network

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INTRODUCTION

Arbuscular mycorrhizal symbiosis (AMS) is an ancient mutualistic relationship between plants and microbes. Coinciding with plant colonization on land, AMS originated approximately 400–450 million years ago (Remy et al., 1994; Redecker et al., 2000; Bidartondo et al., 2011; Rich et al., 2021) and is widespread in over 80% of land plant species from all major lineages (Wang and Qiu, 2006). Arbuscular mycorrhizal fungi (AMF) provide mineral nutrients, such as nitrate and phosphorus, to the host plant in exchange for fixed carbon, including lipids and sugars produced by the host plant (Bago et al., 1999; Vance, 2001; Smith and Read, 2008; Jiang et al., 2017; Luginbuehl et al., 2017). Furthermore, the hyphae of symbiotic fungi can also enlarge the rhizosphere and enhance plant resistance to biotic and abiotic stresses (Miransari, 2010; Santander et al., 2017). The beneficial effects of AMS for plants suggest that this relationship may have helped early land plants adapt to water- and nutrient-deficient terrestrial environments (Choi et al., 2018; Delaux and Schornack, 2021).

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Plants have evolved to precisely regulate the dynamic AMS process (Choi et al., 2018). Since the early studies on *DMI2* (Does not Make Infection 2, also known as *NORK* and *SYMRK*) (Endre et al., 2002; Stracke et al., 2002), over 60 genes involved in AMS have been identified by independent studies (MacLean et al., 2017). The function of a core set of AMS-regulating proteins is highly conserved in angiosperms and even bryophytes (Delaux et al., 2014; Bravo et al., 2016; An et al., 2018; Radhakrishnan et al., 2020; Rich et al., 2021), supporting the ancient origin of the AMS signaling pathway. However, the AMS regulatory network is far from being complete, and new AMS-regulating genes are continuously being reported, including *DLK2*, *ARK2*, and *NPF4.5* (Wang et al., 2020; Ho-Plagaro et al., 2021; Montero et al., 2021).

Recent studies have revealed that the post-transcriptional regulation of mRNAs plays a significant role in AMS regulation. Alternative splicing (AS) and alternative polyadenylation (APA) are key control mechanisms of precursor messenger RNAs (pre-mRNAs), both contributing significantly to increased transcriptome diversity at the post-transcriptional level in eukaryotes (Kalsotra and Cooper, 2011; Elkon et al., 2013). More than 95% and 60% of intron-containing genes in humans and plants, respectively, are alternatively spliced (Pan et al., 2008; Reddy et al., 2013). The distinct transcript isoforms can generate proteins with different functions or that are only present under specific conditions. Recent studies demonstrated that two different isoforms of the gene *Medicago SYNTAXIN 132 (SYP-132)* were generated through AS and APA. One isoform was significantly induced and specifically involved in both nodule symbiosis and AMS, whereas the other was not induced and had no regulatory role in AMS (Huisman et al., 2016; Pan et al., 2016). This example suggests that AS and APA can significantly influence AMS regulation.

Post-transcriptional regulation of mRNAs can also occur through the activity of diverse non-coding RNAs, including microRNA (miRNA), long non-coding RNA (lncRNA), and circular RNA (circRNA) (Song et al., 2019; Yu et al., 2019; Li et al., 2021; Middleton et al., 2021). Several plant miRNAs are key regulators of AMS. MiR171h was the first AMS-regulating miRNA to be identified. It negatively regulates the expression of the symbiotic gene *NSP2* in *Medicago truncatula* and limits excessive AMF colonization (Lauressergues et al., 2012). A subsequent study showed that the inactivation of *M. truncatula* miR396, which targets multiple growth-regulating factor (GRF) genes, enhanced AMF colonization (Bazin et al., 2013). Overexpression of miR393 caused the downregulation of auxin receptor genes and underdeveloped arbuscules (Etemadi et al., 2014). Interestingly, miR171b, which contains a mismatch with the target gene *LOM1*, protects *LOM1* from silencing by other miR171 family members and enhanced AMS in *M. truncatula* (Couzigou et al., 2017). These studies highlight that miRNAs are important regulators of AMS. Although the roles of lncRNAs and cirRNAs in AMS are largely unknown, their involvement in many biological processes is gaining appreciation (Conn et al., 2017; Yu et al., 2019). The competing endogenous RNA (ceRNA) hypothesis posits that lncRNAs and circRNAs with shared miRNA recognition sites can impair miRNA activity through sequestration, thereby functioning as sponges or decoys. Subsequently, this results in the upregulation of miRNA-targeted mRNA expression (Salmena et al., 2011; Thomson and Dinger, 2016). Exploring the transcriptional landscape for different types of non-coding RNAs during AMS may provide new insights into the AMS regulatory network.

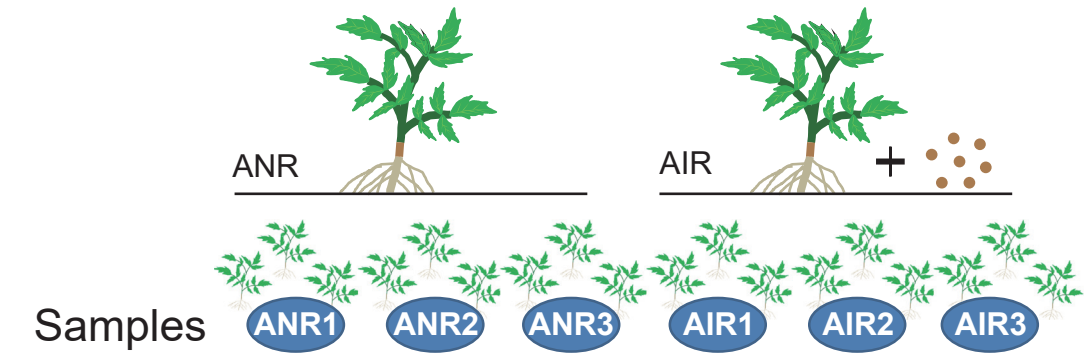
Tomato (*Solanum lycopersicum*) is an emerging model system for AMS studies due to ease of transformation and efficiency in gene editing (Buendia et al., 2016; Shikata and Ezura, 2016; Ho-Plagaro et al., 2019; Liu et al., 2019). To evaluate the potential involvement of different classes of RNAs during AMS, a systematic study on AMS-induced transcriptional alteration in tomato was conducted using multiple transcriptome sequencing approaches, including PacBio single-molecule real-time sequencing (SMRT-seq), RiboMinus Illumina RNA-seq, small RNA sequencing (sRNA-seq), and circRNA-seq. The transcriptional landscape and responsive network of tomato during AMS were comprehensively explored at the RNA level. Results suggest a sophisticated and evolving, RNA layer response during AMS processes and resources generated for this study provide for further, in-depth research of the regulation mechanism of AMS.

RESULTS

A multiple-platform sequencing strategy improves tomato gene annotation for AMS-induced transcriptional reconfiguration analysis

To investigate the root transcriptional landscape in tomato during AMS, both AMF-inoculated (AIRs) and uninoculated (ANRs) root samples were subjected to RNAome analysis (Figure 1). Tomato roots were well colonized by *Rhizophagus irregularis* at six weeks post-inoculation, and there was no significant difference in the fresh weight of shoots between inoculated and uninoculated plants (Supplemental Figure 1). A multiple-platform sequencing strategy was adopted to obtain both protein-coding mRNAs and several different types of non-coding RNA profiles for the root samples. These data were integrated to update annotations of the tomato genome (Figure 1). The annotation for protein-coding genes was improved in several respects by the integration of the SMRT and Illumina RNA-seq data (Supplemental Figures 2 and 3). First, this study annotated 673 novel protein-coding gene loci that had at least one transcript with an ORF of 100 amino acids or longer, 390 of which have annotated domains in the deduced protein sequences and/or were supported by peptide sequences from public proteomic data (Supplemental Table 1). Second, by comprehensive analysis of AS and APA, 14,857 tomato protein-coding genes were found to have more than one transcript in our dataset (Supplemental Data 1 and Supplemental Figure 3). This is a significant enhancement to the reference annotation, which provides only one transcript per gene. Third, the annotation of tomato transcripts regarding the untranslated regions (UTRs) was improved. Fewer than half of the transcripts in the reference genome had UTRs, whereas 98.6% of the transcripts annotated in this study possessed UTRs (Supplemental Figure 2). Further, the annotated UTRs for tomato transcripts identified in this study were significantly longer than those of the reference transcripts (average 1,121

A



sRNA-seq	+	+	+	+	+	+
Illumina-seq	+	+	+	+	+	+
CircRNA-seq	Mix (ANR1, ANR2, ANR3)			Mix (AIR1, AIR2, AIR3)		
SMRT-seq	Mix (ANR1, ANR2, ANR3, AIR1, AIR2, AIR3)					

B

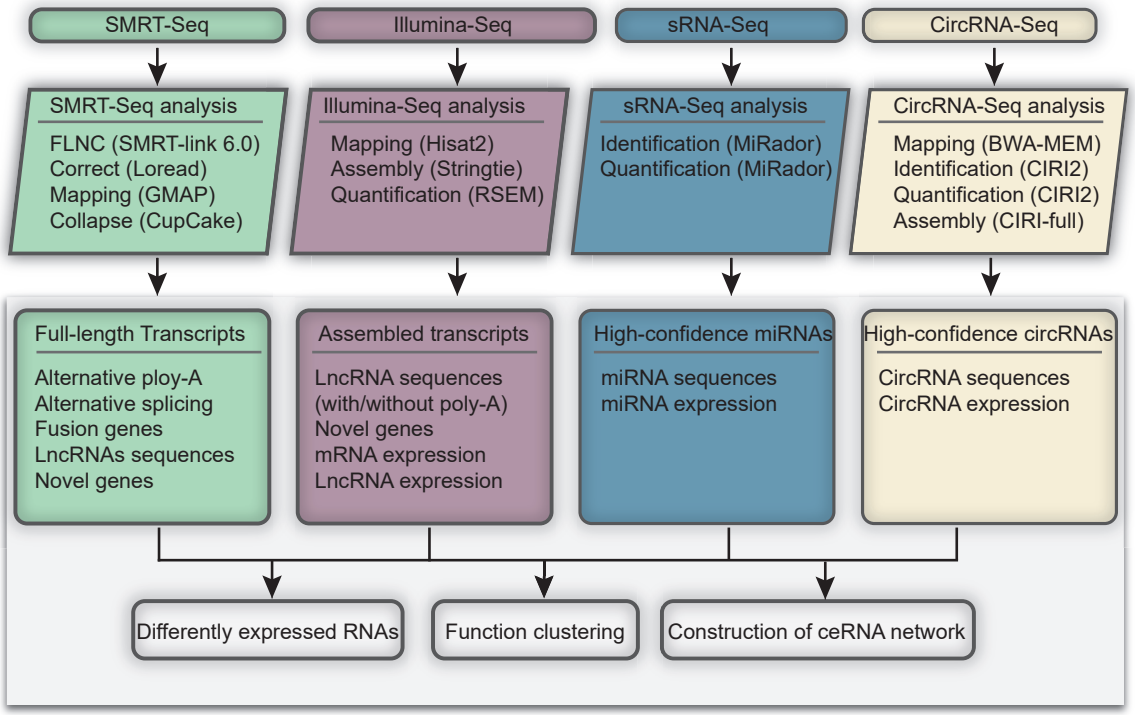


Figure 1. RNAome sequencing strategies and bioinformatics workflow.

(A) Root samples used for different sequence strategies. AIR represents AMF inoculated root samples, and ANR represents AMF uninoculated root samples.

(B) Flowchart of the multi-transcriptome sequencing and analysis strategies.

vs. 492 nt; Supplemental Figure 2). Finally, several types of non-coding RNA were comprehensively annotated by RiboMinus Illumina RNA-seq, sRNA-seq, and circRNA-seq. A total of 5,227 lncRNAs, 140 miRNAs, and 958 circRNAs were identified from the sequencing datasets (Supplemental Data 2, 3, and 4). Overall, the data from multiple sequencing platforms considerably improved the identification and annotation of tomato coding and non-coding gene loci, as well as gene structure, and collectively, these data serve as a reference for the subsequent elucidation of AMS-induced transcriptional response.

AMS-induced transcriptional alteration of thousands of tomato protein-coding genes

The analysis of differentially expressed genes is an important approach for identifying AMS-regulatory genes (Heck et al., 2016; Muller et al., 2019; Ho-Plagaro et al., 2021; Wang et al., 2021; Guo et al., 2022; Hui et al., 2022). To explore transcriptional changes of protein-coding genes of tomato in response to AMS, expression levels from AIR and ANR samples were estimated using mapped Illumina short reads. As a result, 4,707 differentially expressed genes (DEGs) were identified between AIR and ANR samples (Supplemental Table 2), with 3,174 and 1,533 upregulated and downregulated, respectively. Among the upregulated genes (defined as AMS-induced genes hereafter), 28 were orthologous to those known to be involved in AMS, including *PT4*, *RAM1*, *RAD1*, *FatM*, and *STR*, along with some additional AMS-associated genes, demonstrating the AIRs were well colonized by AMF (Supplemental Figure 2 and Supplemental Table 2). Using data from three previously reported tomato AMS-associated transcriptome studies (Sugimura and Saito, 2017; Ho-Plagaro et al., 2019; Tominaga et al., 2022), a comparative analysis with AMS-induced genes was performed. The results showed that a set of 280 AMS-induced genes was identified in all four data sets; 193 and 703 genes were shared by three and two studies, respectively (Figure 2A). There were also 45–2053 genes recognized as AMS-induced genes in only one study, which may be due to differences in the number of AMF spores inoculated, the days after inoculation, and/or plant growth status. In total, 1,176 genes were induced by AMS in at least two independent studies, with this study recovering the largest proportion of them (1,121 genes, 95.3%) as AMS-induced genes among the four independent studies (Figure 2B). By contrast, the other three studies only identified 959 (81.5%), 660 (56.2%), and 365 (31.1%) as AMS-induced genes, suggesting that this report on tomato AMS-induced genes is more comprehensive (Figure 2B). Gene Ontology (GO) enrichment analysis performed on the AMS-induced genes identified in this study revealed that they were significantly enriched in 390 pathways (false discovery rate [FDR] < 0.05; Supplemental Table 3). The representative enriched pathways include transmembrane transport, regulation of protein kinase activity, fatty acid biosynthesis, fatty acid metabolic process, brassinosteroid homeostasis, response to peptide hormone, and ammonium transmembrane transport (Figure 2C). These pathways are highly consistent with those reported by previous studies on AMS using tomato and other angiosperms as host plants (Sugimura and Saito, 2017; Tominaga et al., 2021, 2022; Vasan et al., 2021).

Several recent studies reported that two alternatively spliced variants of the symbiotic gene *SYP-132* have distinct functions in the regulation of AMS (Huisman et al., 2016; Pan et al., 2016). However, a global investigation on the transcript diversity of AMS-induced genes is still lacking. Because multiple transcripts were found for 42% (1,334) of AMS-induced genes from this dataset, the expression level of each transcript in AIR and ANR samples was analyzed. Among them, 472 genes have only one AMS-induced transcript, whereas 303 genes have multiple upregulated transcripts (Figure 2D and Supplemental Table 4). For example, a gene encoding a cytochrome P450 (Solyc04g009930) had three annotated isoforms generated by AS, but only one was significantly induced by AMF colonization (Figure 2E). A similar pattern was also observed for 18 tomato genes that were orthologous to known genes involved in AMS (Supplemental Table 5). However, there are 559 multi-transcript genes that have no AMS-induced transcript, indicating the detected upregulation of these genes is a result of an accumulative effect of multiple transcripts (Figure 2D). These results provide additional clues for evaluating the functional mechanism of AMS-responsive genes demonstrating AS.

A set of tomato AMS-induced genes is conserved among angiosperms

It is assumed that AMS hosts share an ancestral symbiotic signal pathway and regulatory network. To test this assumption, the transcriptomic datasets were mined for conserved cross-species AMS-induced genes from seven additional, phylogenetically diverse angiosperms (Figure 3A). Based on the constructed gene orthologous relationships of 16 angiosperms, including five non-AMS hosts (Supplemental Table 6), 24 conserved orthogroups were identified, each with at least one AMS-induced gene in all AMS hosts analyzed (Figure 3A and Supplemental Table 7). Among them, six orthogroups have known functional AMS signaling or regulatory genes, including *RAD1* (Xue et al., 2015), *RAM1* (Gobbato et al., 2012; Park et al., 2015), *RAM2* (Bravo et al., 2017; Jiang et al., 2017; Luginbuehl et al., 2017), *WRI5a* (Jiang et al., 2018), *SbtM1* (Takeda et al., 2009), and *ARK1* (Roth et al., 2018). The other seven orthogroups encode proteins containing the same domain with known genes involved in AMS. For example, proteins encoded by orthogroups OG0006391 and OG0001767 have the receptor-like kinase domain that is also present in SYMRK (Ried et al., 2014). The cytochrome P450, LysM, germin-like protein, and blue copper protein domains encoded by the orthogroups OG0000230, OG0007943, OG0000063, and OG0001551 are present in the proteins encoded by the symbiotic genes *MAX1*, *CERK1*, *GLP1*, and *Bcp1a/b* (Paradi et al., 2010; Takeda et al., 2011; Carotenuto et al., 2017; Zhang et al., 2018). However, 11 remaining orthogroups did not encode similar domains with known symbiotic genes. These 18 orthogroups offer new candidates for mining for conserved AMS-responsive genes across angiosperms. By conducting a presence/absence analysis of the 24 orthogroups in the genomes of non-AMS hosts, eight were absent from all of the surveyed non-AMS host plants (Figure 3B). These included three orthogroups containing *RAM1*, *RAD1*, and *ARK1*, and five orthogroups that had no known AMS-regulating genes. These evolutionary patterns provide additional evidence to support the involvement of the genes of these orthogroups in AMS.

Additionally, 30, 36, and 49 gene orthogroups that contain AMS-induced genes from six, seven, and five AMS host plants, respectively, were also identified (Supplemental Table 8), suggesting that some accessory AMS-induced genes in angiosperms may have undergone lineage/species-specific gain/loss-of-function. Taken together, 139 orthogroups that contain AMS-induced genes from at least five of the eight AMS host plant species were identified (Figure 3A and Supplemental Tables 7 and 8). Based on the pattern of loss of symbiotic genes in non-AMS hosts, several conserved genes that are potentially involved in AMS have been identified through phylogenomics approaches (Delaux et al., 2014; Bravo et al., 2016). After comparing the

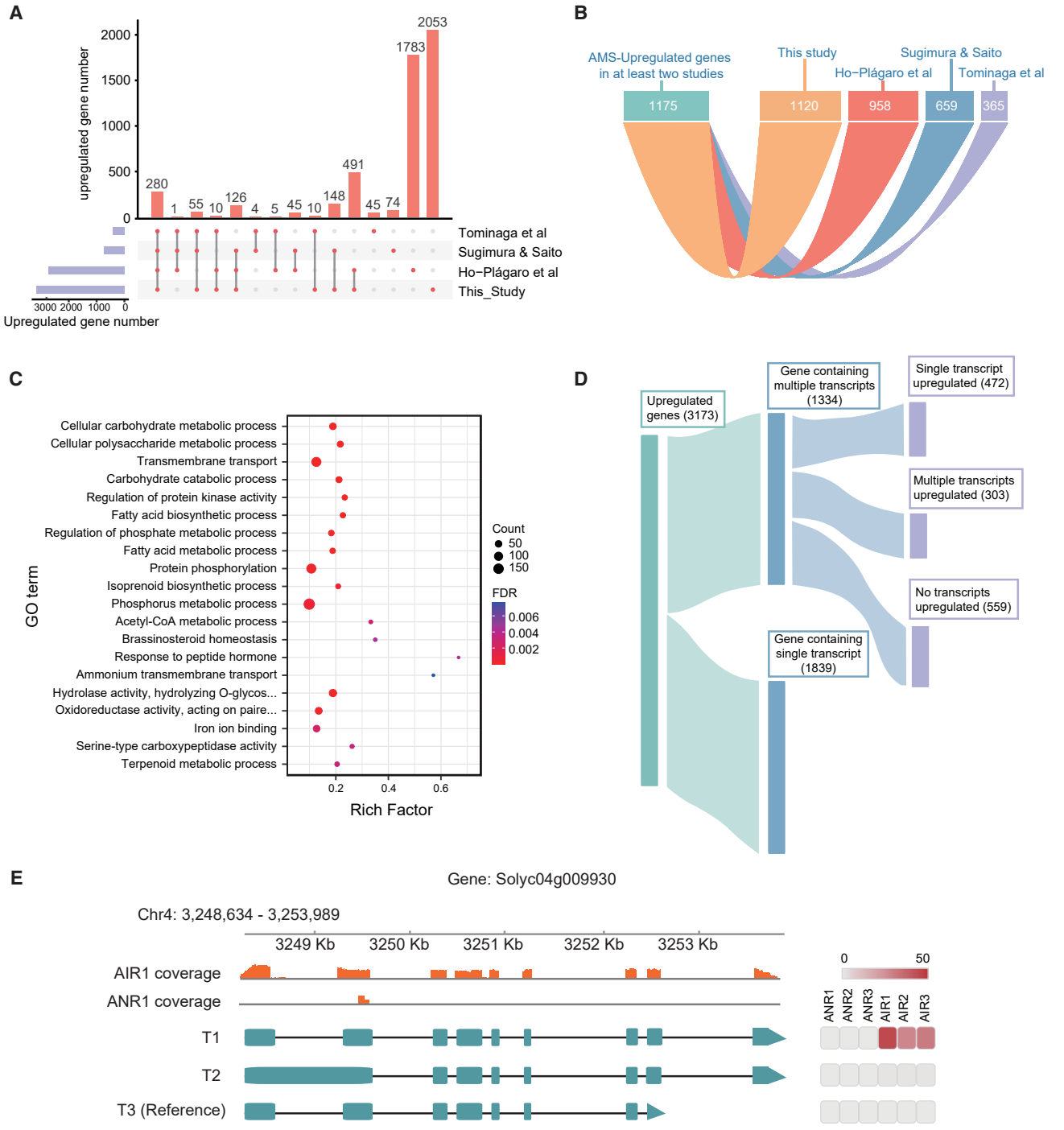


Figure 2. Identification of the upregulated tomato protein-coding genes between AIR and ANR samples.

(A) An UpSet diagram showing the number of genes upregulated in response to AMS among the present study and three previously published studies.

(B) Distribution of AMS-upregulated genes supported by at least two studies among four independent studies.

(C) The representative Gene Ontology (GO) terms of AMS-upregulated genes.

(D) Distribution of AMS-regulated genes containing different numbers of AMS-upregulated transcripts.

(E) An example of a case where only one transcript of a multi-transcript gene (Solyc04g009930) was induced by AMS. The AIR1/ANR1 coverage represents the reads that were mapped to this gene in the AIR1/ANR1 library. T1, T2, and T3 represent three transcript models of this gene, and the heatmap shows their expression pattern in the six libraries.

Figure 3. Conserved AMS-induced orthogroups among angiosperms.

(A) An UpSet diagram showing the number of AMS-induced orthogroups among the eight AMS host plants. The set sizes represent the numbers of AMS-induced orthogroups in 5–8 species. The columns regarding the numbers of orthogroups that are conserved in all angiosperms and all dicot species are indicated by a red star and a green dot, respectively.

(B) Functional annotation of angiosperm- and dicot-scale conserved AMS-induced orthogroups and their distribution in non-AMS host plants. Annotations of orthogroups containing functional AMS-associated genes or encode proteins containing the same domain with known AMS-associated genes are in black font; annotations of orthogroups that did not encode similar domains with known AMS-associated genes are in gray font.

139 orthogroups with previous identified AMS conserved genes, it was found that approximately two-thirds of orthogroups containing AMS conserved genes from the Bravo et al. study, and 14.5% of orthogroups containing AMS conserved genes from the Delaux et al. study overlap with the dataset generated in this study (Supplemental Figure 4). This further demonstrates the conservation of these genes in angiosperm AMS. However, 89 additional orthogroups that have genes induced by AMS in more than five angiosperms were identified in this study. Among the 139 AMS-induced orthogroups, seven had genes that were specifically induced by AMS in all six dicots, but were absent (OG0014063, OG0016346, and OG0016180) or not induced by AMS (OG0004817, OG0012700, OG0004664, and OG0000222) in monocots (Figure 5B). Notably, genes in these orthogroups were also differentially absent in the five non-AMS host plants (Figure 5B). The results suggest that some AMS-responsive modules may have been lost in the monocot lineage or gained in the dicot lineage after the monocot/dicot evolutionary split.

Hundreds of AMS-responsive lncRNAs were annotated from the tomato RNAome

It has been reported that lncRNAs can regulate different biological processes in plants; however, they have not been comprehensively identified and investigated for their potential roles in AMS. In the present study, a total of 5,227 transcripts located in 3,910 loci were identified as putative lncRNAs using four computational tools (Figure 4A and Supplemental Data 2). Of them, 3,718 (71.1%) were single exon transcripts (Figure 4B). This pattern is similar to that observed in oilseed rape, soybean, red clover, and maize (Wang et al., 2016; Chao et al., 2018; Golicz et al., 2018; Yao et al., 2020), suggesting that plant lncRNAs were more likely to be single-exon than protein-coding transcripts. The identified lncRNAs were classified into four types according to their location from the reference annotation, which showed that 43.1% of lncRNAs were from intergenic regions, 43.0% from the sense strand and overlapped with protein-coding genes, 8.5% from the antisense strand of protein-coding genes, and 5.4% from intronic regions (Figure 4C). A BLASTN was used to search candidate lncRNA homologs to known tomato lncRNAs from the CANTATAdb database (Szczesniak et al., 2019). The results showed that 2,020 (38.65%) of the 5,227 identified putative lncRNAs have been documented by the CANTATAdb in the tomato lncRNA catalog, and the others were newly reported by this study (Figure 4D). The mean length of lncRNAs from this study was 1,135 nt, ranging from 200 to 15,073 nt, which is slightly longer than documented tomato lncRNAs in the CANTATAdb (Figure 4E).

Expression analysis revealed that 587 tomato lncRNAs were differentially expressed (DE) between AIR and ANR samples (Figure 4F and Supplemental Table 9). Of these, 307 were upregulated and 280 were downregulated. By surveying the parental gene loci of these DE lncRNAs, 316 were found from gene loci that have multiple transcripts (Supplemental Data 2), suggesting that AS also occurs at non-coding gene loci. Through various mechanisms, lncRNAs can activate or repress the expression of neighboring genes (Sun and Kraus, 2015). To explore the potential regulatory functions of tomato AMS-responsive lncRNAs, their expression patterns were compared with mRNAs that were transcribed from adjacent or overlapping genes. Of these, 109 potential target mRNAs showed significant expression alteration, and 36, 21, and 52 were targeted by lncRNAs from intergenic regions, antisense strands, and sense strands, respectively. Most of the lncRNA-mRNA pairs (i.e., 26 intergenic lncRNA-mRNA, 19 antisense lncRNA-mRNA, and 49 sense lncRNA-mRNA) showed positive correlations in expression (Figure 4G), suggesting that a large proportion of lncRNAs may play positive roles in the expression of neighboring mRNAs. Among these lncRNA targets, two mRNAs transcribed by Solyc07g006610 and Solyc04g050940, were orthologous to *M. truncatula* symbiotic genes *KIN2* and *KIN3*, respectively (Supplemental Table 5) (Yang et al., 2012; Bravo et al., 2016).

Analysis of AMS-responsive miRNA-target gene pairs

A total of 140 miRNAs were identified from the six datasets, 95 of which belong to 30 known tomato miRNA families in the miRbase. The others represent putatively novel miRNAs (Supplemental Data 3). Specifically, three miRNA families identified in this study, miR165, miR1446, and miR8025, have not been documented in the tomato miRNA catalog by miRBase. Among the known miRNA families, the number of members in each miRNA family ranged from one to 13 (Figure 5A). The length of the miRNAs ranged from 20 to 24, with the majority (96 miRNAs, ~68.6%) being 21 nt (Figure 5B).

Ten miRNAs, nine known and one novel (miRnovel22), showed significant differential expression between AIR and ANR samples (Figures 5C and Supplemental Table 5). Among these, three from different families (miR319, miR390, and miR9470) were upregulated upon AMF colonization. By contrast, seven miRNAs from four families (miR166, miR399, miR482, and miRnovel22) were downregulated. Results from qPCR analysis confirmed these differences (Supplemental Figure 6). Prediction targets of DE miRNAs against all tomato transcripts revealed that 324 transcripts from 250 genes oppositely showed significant differential expressionfrom the miRNAs targeting them after AMF colonization (Figure 5C). However, only 72 transcripts from 72 genes in the reference genome were predicted as targets of these miRNAs with the same criterion. The larger number of predicted targets is a result of using the improved annotation, which includes multiple isoforms from the same gene loci and extended UTRs. Specifically, among the 324 predicted target transcripts, 43 were targeted by miRNAs at the UTR. However, only 23 could be predicted as targets of DE miRNAs if the reference annotation was used. For example, a DUF4228 domain-containing gene, the orthologs of which were induced in all eight AMS hosts during AMF colonization and was absent from all surveyed non-AMS host plants (Supplemental Table 7), was predicted to be targeted by miR482e-5p at the 3′ UTR of the updated annotation. However, it was not predicted as the target when using the reference annotation (Figure 5D). Finally, the data showed that transcripts from three tomato genes orthologous to *RAD1* (Solyc03g110950), *HA1* (Solyc08g078200), and *NOPE1* (Solyc03g080020) were targeted by AMS-induced miR166a, miR166d, and miR482f, respectively (Supplemental Table 5). The GO enrichment analysis showed that the functions of predicted miRNA targets were mainly enriched in the terpenoid biosynthetic process, ATP hydrolysis activity, lipid biosynthetic/metabolic process,

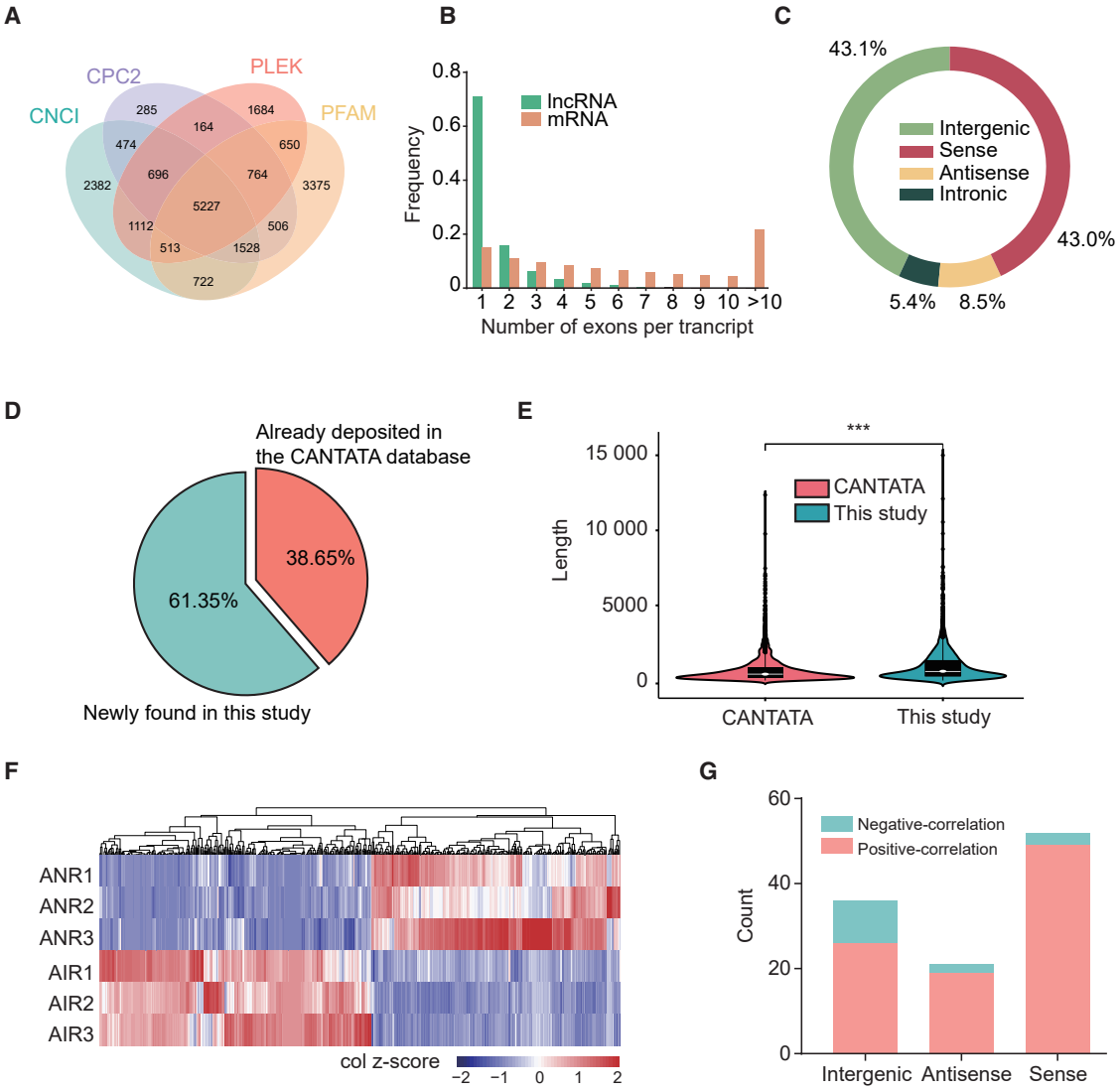


Figure 4. Identification of tomato lncRNAs from AIR and ANR samples.

(A) Venn diagram of lncRNAs predicted by CNCI, CPC2, PLEK, and PFAM.

(B) Comparison of the number of exons of lncRNAs and mRNAs.

(C) Proportion of four different types of lncRNAs.

(D) Comparison of the lncRNAs identified in this study with tomato lncRNAs in the CANTATA database.

(E) Length difference of lncRNAs identified in this study with those in the CANTATAdb database. Statistical analysis was performed with Student's *t*-test (***$p < 0.001$).

(F) Heatmap of differentially expressed lncRNAs.

(G) Histogram showing the counts of positive or negative correlations between DE lncRNAs and their overlapping or adjacent mRNAs.

and gibberellin metabolic process (Figure 5E), suggesting that tomato miRNAs may have been involved in AMS regulation by modulating nutrient metabolism and signal transduction.

Identification of tomato circRNAs and their involvement in AMS competing endogenous RNA network

To explore the role of circRNAs in tomato AMS regulation, three detection tools, Find_circ, CIRI2, and CIRCexplorer2, were used to identify circRNAs from the two libraries, each of which was pooled with three AIR or three ANR samples. Collectively, a total of 9,043 unique circRNAs were predicted from the two libraries using the three tools (Supplemental Figure 7). Among these, 958 were detected by all three tools (Supplemental Data 4), suggesting that these are high-confidence circRNAs. After cross-referencing these circRNA sequences with PlantcircBase (Chu et al., 2017), 574 were found to be homologous with tomato circRNA sequences already in the database, with the remaining 384 being newly identified. The length of the high-confidence circRNAs ranged from 197 to 27, 820 nt, with more than half being <2,000 nt (Figure 6A). Most of the high-confidence circRNAs were lowly expressed in both libraries with ~60% having <5 junction reads (Figure 6B). By contrast, <10% high-confidence circRNAs had >30 junction reads (Figure 6B). Comparing the number of reads at the back-spliced junction site revealed that 88 circRNAs were significantly

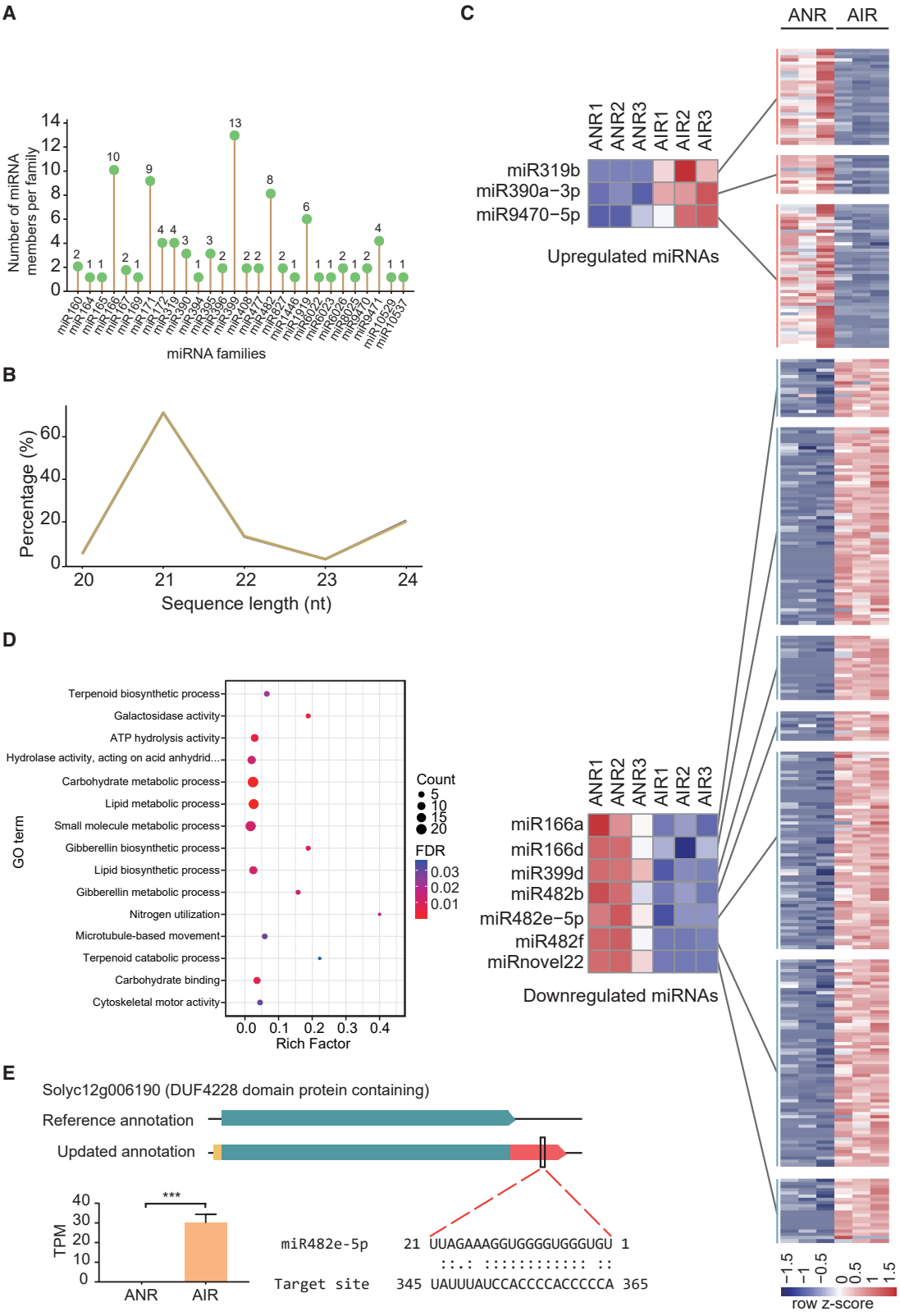


Figure 5. Identification of tomato AMS-responsive miRNAs.

(A) Number of members of each miRNA family identified from this study.

(B) Length distribution of miRNAs.

(C) Heatmap of differentially expressed miRNAs and their corresponding target mRNAs.

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induced upon AMF colonization, whereas 58 were significantly down-regulated (Figure 6C). These DE circRNAs are candidate regulators of AMS. A significant positive correlation between the expression pattern of most circRNAs and their parent genes has been reported (Ye et al., 2015). The linear transcripts from the parental genes of 31 DE circRNAs showed significant alteration in expression, 24 of which were positively correlated with DE circRNAs, and seven were negatively correlated (Figure 6D). Among these parental genes, three of them are orthologs of known AMS conserved genes (i.e., *CCD8* [Solyc08g066650], *CYT733A1* [Solyc04g071150], and *HA1* [Solyc08g078200]) (Supplemental Table 5).

A ceRNA network analysis was performed to predict the interactive relationship among miRNAs, mRNAs, lncRNAs, and circRNAs during AMS (Figure 6E). The analysis included 10 miRNAs (3 up/7 down), 587 lncRNA (307 up/280 down), 146 circRNA (88 up/58 down), and 4,248 mRNA (2,170 up/2,078 down). All DE mRNAs, DE lncRNAs, and DE circRNAs that showed significant differential expression in an opposite direction from the DE miRNAs upon AMF colonization were subjected to miRNA target prediction. The ceRNA network showed that the 10 DE miRNAs could target 331 RNAs of different types, including 312 mRNAs, 12 lncRNAs, and seven circRNAs, forming 343 miRNA-mRNA,12 miRNA-lncRNA, and nine miRNA-circRNA pairs (Figure 6E and Supplemental Tables 10 and 11). Among the 10 DE miRNAs, eight had noncoding RNA targets in addition to the mRNA targets, suggesting RNAs as potential sponges or decoys to fine-tune the function of miRNAs. Notably, one miRNA may target multiple targets from the same or different RNA types, whereas one mRNA, lncRNA, or circRNA can be targeted by several miRNAs. Among the 10 DE miRNAs, miR482f had the greatest number of predicted targets, including 65 mRNAs, four lncRNAs, and four circRNAs. One of the circRNAs (SL3.0ch06:2 043 151|2 046 391) showed interaction with two miRNAs (miR482e-5p and miRnovel22), suggesting a pleiotropic role by binding multiple miRNAs to regulate the expression of a large number of mRNAs. Taken together, these data demonstrate that the ceRNA network of noncoding RNAs plays a significant role in AMS by connecting symbiotic genes into a highly regulated network.

Construction of a tomato symbiotic transcriptome database

To make the RNAome data obtained in this study accessible to researchers in the symbiotic field, a tomato symbiotic transcriptome database (TSTD, https://efg.nju.edu.cn/TSTD/) was established (Figure 7). The database not only collects the expression data of protein-coding and non-coding genes from AMF colonized and uncolonized tomato roots but also provides the orthologs of tomato protein-coding genes in 15 other plants from basal angiosperm, dicot, monocot, magnoliids, and ceratophyllum lineages. Notably, the expression data of tomato orthologous genes from seven other AMS hosts were also included in the database to assist in online comparative analysis and find conserved, cross-species AMS-induced genes.

The major functions of the database include: (1) browsing AMS-induced tomato protein-coding genes and noncoding RNAs, (2) searching the tomato protein-coding genes by the BLAST program with a protein sequence to obtain the expression patterns of homologous genes, (3) searching for orthologs across 16 angiosperms and their AMS-induced expression patterns using a gene of interest, (4) retrieving cross-species shared AMS-induced orthogroups by choosing species of interest, and (5) batch downloading datasets of interest.

DISCUSSION

Recognizing core and accessory symbiotic genes by expression and evolutionary features suggests the continuous evolution of the angiosperm AMS-responsive network

The colonization of the terrestrial environment by plants was facilitated by an ancient mutualism with AMF. In the present study, a comparative transcriptomic analysis of tomato, along with seven AMS host plants from the angiosperm monocot and dicot lineages, revealed 24 conserved AMS-induced gene orthogroups (Figure 3A). This result supports the concept of an ancient and highly conserved symbiotic pathway (Radhakrishnan et al., 2020; Rich et al., 2021). Notably, these orthogroups not only included several known AMS regulation genes, including *SbtM1* (Takeda et al., 2009), *RAD1* (Xue et al., 2015), *RAM1* (Gobbato et al., 2012; Park et al., 2015), *RAM2* (Bravo et al., 2017; Jiang et al., 2017; Luginbuehl et al., 2017), *WRI5a* (Jiang et al., 2018), and *ARK1* (Roth et al., 2018), but also revealed 18 novel orthogroups that may be involved in AMS regulation. Among them, genes from seven orthogroups encode proteins with the same domain with known symbiotic genes *MAX1*, *CERK1*, *GLP1*, and *Bcp1a/b* (Paradi et al., 2010; Takeda et al., 2011; Carotenuto et al., 2017; Zhang et al., 2018), suggesting that these AMS-responsive candidates may be involved in symbiotic pathways similar to their paralogs with known functions. The remaining orthogroups did not encode similar domains with known symbiotic genes. The functional annotation revealed that several of them are transcription factors and enzymes that utilize different substrates (Figure 3B). Functional characterization of genes in these orthogroups may help to identify novel cross-species, conserved AMS-responsive modules. Four of the newly identified orthogroups, three encoding DUF538, DUF4228, and F-box/kelch-repeat plant domains, and one annotated as a taurine catabolism dioxygenase, are notable because of their absence in all non-AMS host plants (Figure 3B). This evolutionary pattern has been observed in *RAD1* (Xue et al., 2015) and *RAM1* (Park et al., 2015), and has been used as a criterion to mine for new AMS regulating genes (Bravo et al., 2016). This suggests that these genes in the four orthogroups are strong candidates for AMS regulation. Elucidating the role of genes from the above

(D) The representative GO terms of target genes of DE miRNAs.

(E) An example showing that the gene Solyc12g006190 was predicted to be targeted by miR482e-5p at the 3′ UTR when using updated annotations, but it could not be predicted as a target gene using the reference annotation. Histogram showing the TPM of the Solyc12g006190 gene in AIR and ANR samples.

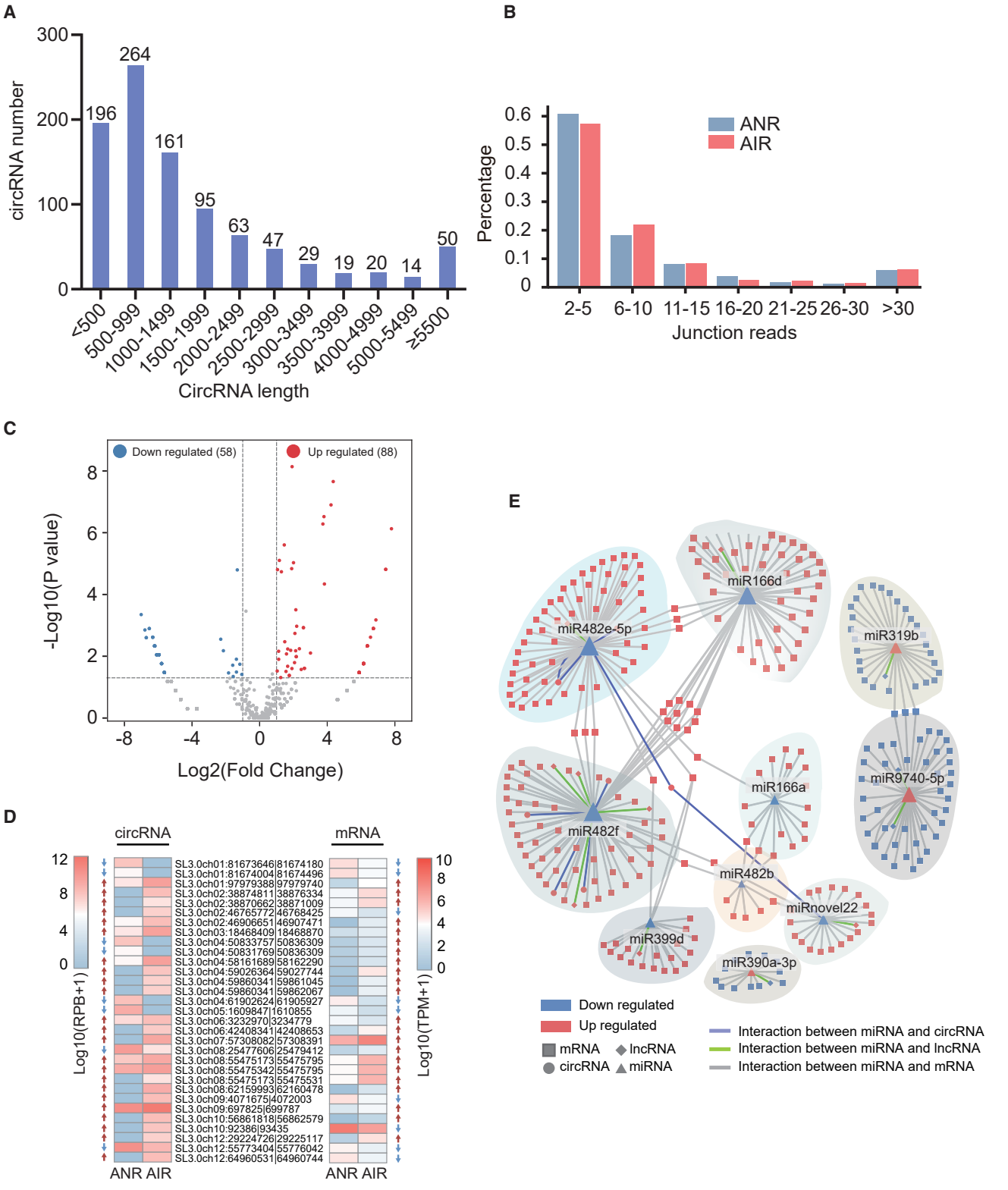

Figure 6. Identification of tomato AMS-responsive circRNAs.

(A) Length distribution of circRNAs.

(B) Percentage of back-spliced junction reads.

(C) Volcano plot showing differently expressed circRNAs between AIR and ANR samples.

(legend continued on next page)

18 orthogroups in AMS regulation would extend the gene list of the AMS core pathway that is shared by angiosperms.

Apart from the core symbiotic genes with conservative roles in AMS regulation across different species, there are also genes involved in AMS regulation in one species but are absent from or have lost their function in other AMS-host species. For example, *MIG1* is induced by AMF fungi inoculation and can control root cortical cell expansion by intersecting with *DELLA1* in the dicot species *M. truncatula* (Heck et al., 2016; Seemann et al., 2021). However, its ortholog is absent in the monocot, rice. Moreover, Radhakrishnan et al. (2020) surveyed >30 genes with symbiotic functions across land plant species and found that more than half were absent from at least one AMS host species. These results suggest that some of the symbiotic genes may have accessory roles in AMS regulation, and functional deficiency of these genes can be compensated for by the evolution of an alternative pathway or through the modulation of the preexisting pathway in the host plants. In this study, 115 orthogroups that contain AMS-induced genes from at least five of the eight AMS host species were identified (Figure 3A), suggesting that a large number of accessory symbiotic genes may exist in the AMS regulatory network, and thus indicating that the AMS-responsive network is undergoing continuous evolution in angiosperms.

Notably, among the accessory orthogroups, seven have genes that were specifically induced by AMS in all six dicots but were absent from or showed no induction in expression in monocots (Figure 3B). All but one of these orthogroups are present in the genome of the basal angiosperm, *Amborella trichopoda* (Supplemental Table 6), suggesting that the AMS-responsive network may have been reshaped in the monocot lineage after its separation from the dicot lineage through loss of some ancestral symbiotic genes. Monocot and dicot species have dramatic differences in root structure (Smith and De Smet, 2012). The divergence of the AMS-responsive network between monocot and dicot species thus may be a result of adaptive evolution of plants to fine-tune this symbiotic relationship between the two partners. A recent transcriptomic study of *Poncirus trifoliata* upon AMF inoculation revealed fewer AMS-responsive genes when compared to other herbaceous plants (An et al., 2018), suggesting that root structure variation may have a role in the evolution of AMS-responsive networks. This study presents a significant number of candidates to extend the core symbiotic gene list and proposes that the continuous evolution of accessory symbiotic genes in angiosperms has reshaped the AMS-responsive network during speciation.

An integrated map of AMS-induced RNAome landscape suggests sophisticated RNA layer AMS regulation

While the post-transcriptional regulation of gene expression has been documented in many biological processes in plants (Laloum et al., 2018; Szweykowska-Kulinska and Jarmolowski, 2018), its role in AMS has been less well explored. The Micro Tom tomato was used as a model to uncover the RNAome reconfiguration induced by AMS to elucidate the RNA layer regulation of AMS. Although a reference genome for tomato is available (Tomato Genome, 2012), and several previous studies have reported on tomato transcriptomics during AMS (Sugimura and Saito, 2017; Ho-Plagaro et al., 2019; Tominaga et al., 2022), a global model on the role of non-coding RNA function during tomato AMS is still lacking. The results from this in-depth, combinatorial RNA-seq study of AIR and ANR samples presented here not only significantly improved the annotation of the tomato genome, but also provided a more comprehensive understanding of AMS-induced protein-coding genes (Supplemental Figures 2 and 3). Finally, it provided an in-depth analysis of non-coding RNA species affecting AMS gene regulation including 140 miRNAs, 5227 lncRNAs, and 958 circRNAs. Of these, 45 miRNAs, 3207 lncRNAs, and 384 cirRNAs were newly identified in this study.

The improved annotation of the tomato genome enabled the elucidation of the complexity of the tomato RNAome and provided a global landscape of AMS-induced RNAome reconfiguration in tomato. The post-transcriptional processing of pre-mRNAs contributes significantly to transcriptomic diversity (Abdel-Ghany et al., 2016). While there are a couple of studies that have demonstrated the involvement of AS in symbiotic gene function (Huisman et al., 2016; Pan et al., 2016), the results from this study are significantly more comprehensive. In total, it was shown that 1,334 of the AMS-induced genes in tomato have multiple transcripts, including 472 genes with only one AMS-induced transcript (Figure 2D). These results provide additional in-depth insight into the functional mechanism of AS of AMS-responsive genes and for the determination of the functional transcripts involved in AMS regulation.

The function of several miRNAs in regulating the formation and development of arbuscules has been demonstrated experimentally in the legume *M. truncatula* (Lauressergues et al., 2012; Bazin et al., 2013; Etemadi et al., 2014; Couzigou et al., 2017). Recently, miRNAs that are responsive to AMS in non-legume plants have been reported, including maize and *Nicotiana attenuata* (Pandey et al., 2018; Xu et al., 2018). The results presented here showed that 10 miRNAs exhibited significant differential expression upon AMF inoculation in tomato. Among them, miR319b was expressed in all three biological replicates of the AIR samples, but was not detected in the ANR samples (Figure 5C). Previous studies reported that miR319 could target *TCP* genes in *Arabidopsis*, cotton, and the common bean (Palatnik et al., 2003; Formey et al., 2015; Cao et al., 2020). The overexpression of miR319 resulted in a reduced root length:width ratio and increased rhizobial inoculation in the rhizobia symbiosis of the common bean (Martin-Rodriguez et al., 2018). The miR166 is another miRNA family that has been shown to regulate rhizobia symbiosis by targeting the class III homeodomain-leucine zipper (*HD-ZIP III*) genes (Boualem et al., 2008). This study revealed that two

(D) Heatmap of the circRNAs and their parental gene expression.
(E) A competing endogenous RNA network of tomato during AMS. Different types of RNA are represented by different shapes, while their upregulation and downregulation between AIR and ANR samples are indicated by different colors. Predicted interactions between different types of RNAs are indicated by colored lines.

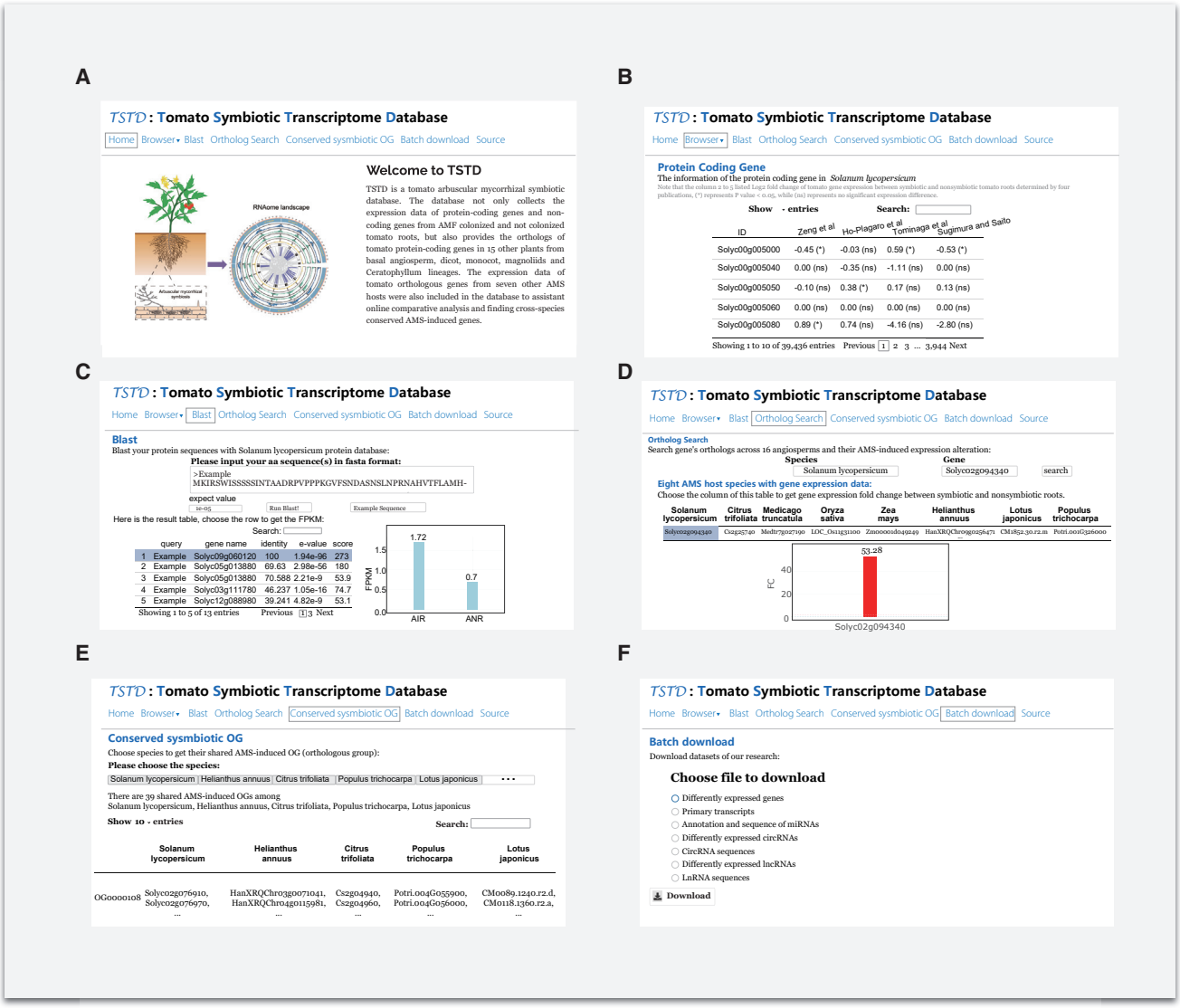

Figure 7. Construction of a tomato symbiotic transcriptome database (TSTD).

Representative screenshots of the TSTD interface showing the major functions of TSTD: **(A)** homepage, **(B)** gene expression browser, **(C)** BLAST tomato genes using protein sequences to obtain its expression, **(D)** search a gene to obtain its orthologs from 16 angiosperms and the expression fold change upon AMF colonization, **(E)** browser AMS-induced orthologroups shared by different combinations of AMS hosts, and **(F)** batch download datasets of interest.

miR166 members, miR166a and miR166d, are downregulated during AMS. Interestingly, two tomato orthologs of *RAD1* and *HA1* were predicted to be the targets of miR166a and miR166d, respectively. The AMS-induced differential expression of miR319 and miR166 members suggested that these might be candidate regulatory miRNAs of AMS in tomato. In addition, miR399 showed significant downregulation in tomato AIR samples, as well as a similarly downregulated expression pattern in maize and *N. attenuata* upon AMF inoculation (Pandey et al., 2018; Xu et al., 2018). This suggests that miR399 may be a conserved miRNA in AMS regulation across monocots and dicots. Phosphate (Pi) is the most important mineral element delivered by AMF to the host plant. Previous studies have shown that miR399 responds to stress induced by low phosphorus in monocot and dicot plants (Fujii et al., 2005; Bari et al., 2006; Chiou et al., 2006; Du et al., 2018; Sega et al., 2021). The results suggest that miR399 may be involved in AMS regulation by balancing Pi uptake pathways. Three lncRNAs with low sequence similarity in *Arabidopsis* and maize, *AtIPS1*, *AtAT4*, and *ZmPILNCR1*, could inhibit miR399-directed cleavage of its target gene *PHO2* (Franco-Zorrilla et al., 2007; Du et al., 2018). Interestingly, a lncRNA in tomato was also predicted to be targeted by miR399 and was negatively correlated with miR399 in expression upon AMF inoculation. However, sequence similarity of the lncRNAs from these species was not found in tomato, suggesting that a similar regulation mechanism of miRNA399 involving lncRNA may have independently evolved in the three species. These examples also support the conserved and continuously evolving AMS-responsive network from the RNA layer. The

results suggest possible important roles for these miRNAs in AMS, although future experiments are needed to verify the interactions with corresponding target genes.

miRNAs from six families have been shown to respond to AMS in tomato after being incubated with AMF for 11 weeks (Wu et al., 2016). Four of them also have members that show differential expression in the 6-week symbiosis study conducted here, yet they did not reach the criterion (as described in Materials and Methods) as AMS responsive. This may be due to differences in genetic background of the host plants, the number of AMF spores inoculated, and/or the days after inoculation and plant growth status used by independent studies. This result could also be seen when comparing AMS-responsive miRNA repertoires among tomato, maize, and *N. attenuata* (Pandey et al., 2018; Xu et al., 2018), which only recovered one common AMS-responsive miRNA. Such a pattern could also be observed in AMS-induced protein-coding genes, which showed that a considerable number of tomato AMS-induced genes were only detected by one of the four studies (Figure 2). Both cases suggest high dynamic expression of coding and non-coding RNAs during AMS.

To our knowledge, there are currently no studies that have experimentally demonstrated the regulatory role of lncRNA and circRNA in AMS, mainly due to the lack of knowledge of their existence and expression patterns during AMS. A total of 587 differentially expressed lncRNAs were found upon AMF colonization, and the expression of 24 lncRNAs in the AIR was >1,000-fold higher compared to ANR, suggesting that these lncRNAs are highly likely to be involved in the regulation of AMS (Supplemental Table 9). Using a circRNA-seq strategy, >900 high-confidence cirRNAs were identified, among which were 146 AMS-responsive. Notably, among 55 tomato genes that are orthologs to known genes involved in AMS, six were predicted to be regulated by DE lncRNA or DE circRNAs (Supplemental Table 5), suggesting lncRNAs and circRNAs may be involved in the regulation of some key genes in the symbiosis-related pathway. The ceRNA hypothesis proposes that lncRNA and circRNA can target the expression of mRNAs with common miRNA recognition elements by regulating miRNA activity (Thomson and Dinger, 2016). Taking these datasets as input, an AMS ceRNA network including 10 miRNAs, 587 lncRNAs, 146 circRNAs, and 4,247 mRNAs was constructed. These results provide new insights into the function of non-coding RNAs and complex network in AMS regulation and can serve as a resource for exploring the regulation mechanism of AMS-associated protein-coding genes.

In summary, the present study illustrates RNAome dynamics in tomato during AMS and provides a global RNAome landscape for future studies of AMS regulation, encompassing AS, APA, miRNAs, lncRNAs, and circRNAs. A set of conserved angiosperm gene orthogroups and numerous accessory orthogroups induced by AMF in AMS host plants were identified. In addition, a tomato symbiotic transcriptome database (TSTD) was constructed by integrating coding and non-coding AMS-responsive RNAs. These results will serve as a basic resource for guiding future functional studies on the AMS-responsive network at both the protein and RNA levels.

MATERIALS AND METHODS

Plant growth and harvesting

Tomato (*S. lycopersicum* cv. 'Micro Tom') seeds were surface-sterilized and germinated on half-strength Murashige and Skoog (MS) basal medium with sucrose and phytagel at 24°C. After germination, seedlings were transferred to plastic pots filled with a mixture of sterilized sand/gravel (1:1 ratio). For mycorrhizal treatment, each plant was inoculated with 100 spores of *R. irregularis* (strain DAOM197198) freshly extracted from carrot hairy root co-cultures and grown in a climate-controlled growth room with 16 h light at 24°C and 8 h dark at 22°C. Plants were watered weekly with half-strength Hoagland's solution containing 20 μM phosphates. Roots were harvested at six weeks post-inoculation. Mycorrhization rate was calculated as described previously (Mcgonigle et al., 1990). Briefly, 40–50 root fragments (~1 cm) were placed on a microscopic slider with 1-cm grids. Greater than 120 intersections of the roots and grids per slide were observed using a TE2000-U microscope (Nikon, Tokyo, Japan).

Library preparation and RNA-seq

Six root samples, including three AIRs with an AMF colonization rate of 60%–70% and three ANRs, were used for the analysis. Each sample was composed of roots from two to three plants. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) for each sample. For SMRT-seq, total RNA from six root samples was pooled together in equal amounts. The first cDNA strand was synthesized using the SMARTer PCR cDNA Synthesis Kit (Takara, https://www.takarabio.com/) from purified polyA(+) RNA. The product was sequenced using SMRT-seq on the PacBio Sequel platform at Novogene (Beijing, China).

For Illumina sequencing, cDNA libraries were constructed using rRNA-depleted RNA via a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA). For sRNA-seq, sRNA library preparation was performed using a Small RNA Sample Prep Kit (Illumina, San Diego, CA, USA). For circRNA-seq, rRNA-depleted circRNA libraries after RNase R treatment were constructed using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). The libraries (Illumina-seq, 2 × 150 bp; sRNA-seq, 1 × 50 bp; and circRNA-seq, 2 × 150 bp) were sequenced on the Illumina HiSeq X platform at Novogene.

PacBio data analysis

PacBio subreads were processed using the SMRT-Link 6.0 software (http://www.pacb.com/) to generate full-length, non-full-length, and chimeric reads. Consensus isoforms were identified using the ICE algorithm from full-length non-chimeric (FLNC) sequences and were further polished with non-full-length reads and subreads to obtain high-quality isoforms using Quiver. Full-length reads were further error-corrected using LoRDEC v0.9 (Salmela and Rivals, 2014) together with Illumina short reads. The corrected consensus transcripts were then mapped to the *S. lycopersicum* reference genome (https://phytozome.jgi.doe.gov/pz/portal.html) using GMAP v2020-06-30 (Wu and Watanabe, 2005). The transcripts located in certain gene loci were then collapsed into non-redundant consensus isoforms using *collapse_isoforms_by_sam.py* from the Cup_Cake v12.2.0 package (https://github.com/Magdoll/cDNA_Cupcake).

Illumina data analysis

Clean reads from this study and public databases (Sugimura and Saito, 2017; Ho-Plagaro et al., 2019; Tominaga et al., 2022) were aligned to the *S. lycopersicum* reference genome using Hisat2 v2.1.0 (Kim et al., 2019). The obtained transcripts were assembled by using StringTie v1.2.3 with the parameters '-p 8 -j 5 -a 20 -f 0.8' (Pertea et al., 2015). RSEM was used to calculate the isoform level expression in terms of TPM (transcripts per million) with the parameters '–bowtie2 –paired-end -no-bam-output' (Li and Dewey, 2011). The read counts were

processed with featureCounts v2.0.1 to quantify gene expression and normalized using the trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010; Liao et al., 2014). The different expression levels of genes were assessed using the EdgeR package based on FDR-adjusted $P < 0.05$ and |log2fc|>1 (Robinson et al., 2010). The GO term enrichment was performed using ShinyGO v0.76 with default parameters (Ge et al., 2020).

Identification of novel protein-coding genes

Gene loci from the PacBio and Illumina mapping results were compared with the annotations from the tomato reference genome by using gffcompare v0.12.1 (Pertea and Pertea, 2020). The loci that did not overlap with an annotated gene in the reference genome and with at least one transcript with an open reading frame (ORF) length >100 amino acids were considered to be novel protein-coding genes. HMMscan v3.3.2 was used to predict the domains encoded by the transcripts of novel protein-coding genes against the Pfam-A database (Finn et al., 2014). Peptides of tomato proteomic data retrieved from PRIDE Archive database (Proteomics identifications database, https://www.ebi.ac.uk/pride/archive/) were mapped to protein sequences encoded by the novel genes to validate their translation (Perez-Riverol et al., 2022).

Construction of gene orthologous relationships among angiosperms

The gene orthogroups for 16 angiosperm species (Supplemental Table 12), including eight species with available expression data during arbuscular mycorrhiza symbiosis, were analyzed using OrthoFinder v2.5.2 with the parameter '-M msa' (Emms and Kelly, 2019), as described by Rich et al. (2021).

Characterization of AS and APA events

The GTF annotation file assembled from the Illumina and PacBio data was used for the identification of AS events using the AStalavista v3.2 tool with the parameter '-t asta' (Foissac and Sammeth, 2007). The TAPIS v1.2.1 pipeline was applied to detect APA sites (Abdel-Ghany et al., 2016). MEME-chip v5.4.1 (https://meme-suite.org/meme/tools/meme-chip) analysis based on upstream sequences of poly(A) sites was used to detect poly(A) sequence signals (Machanick and Bailey, 2011).

Identification of lncRNAs

CPC2 v0.1, CNCI v2, PLEK v1.2, and PFAM v32 were used for predicting the protein-coding potential of transcripts generated from the Illumina and SMRT data (Kong et al., 2007; Sun et al., 2013; Finn et al., 2014; Li et al., 2014). The criteria for candidate lncRNAs were set as follows: (1) the longest representative transcript had no ORF >100 amino acids, and (2) its nucleotide sequence was at least 200 nt.

miRNA data analysis

miRNAs were identified using miRador (https://github.com/rkweku/miRador) with the options 'gap = 6, match = 3, mismatch = -4, threshold = 40, maxRepLen = 300, organism = Sly' (Hammond et al., 2021). Candidate miRNAs were annotated as known or novel miRNAs by referencing the latest miRBase v22.1 (Kozomara et al., 2019). The miRNAs with |log2fc| >1 and $p < 0.05$ were considered to be AMS-responsive miRNAs. The psRNATarget v2 was used to predict miRNA targets with default parameters. The ceRNA network was visualized using Cytoscape v3.8.2.

CircRNA data analysis

Clean reads were mapped to the *S. lycopersicum* reference genome using BWA-MEM v0.7.12 software with the parameter '-T 19' (Li and Durbin, 2009; Langmead and Salzberg, 2012). CIRI2 (v2.0.6), CIRIexplorer2 (v2.4.0), and Find_ciric (v1.2) were combined to detect potential back-splice sites (Memczak et al., 2013; Zhang et al., 2016; Gao et al., 2018). Reconstructions of full-length circRNAs were achieved using CIRI-full (Zheng et al., 2019).

Quantitative real-time-PCR

The expression of miRNAs was measured by quantitative real-time PCR. cDNA synthesis was performed using a stem-loop primer with the miRNA 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). Quantitative real-time–PCR was performed using a CFX96 real-time system (Bio-Rad, Hercules, CA, USA) with the miRNA Universal SYBR qPCR Master Mix (Vazyme). The tomato *U6* gene was used as an internal control. All of the primers used are listed in Supplemental Table S13.

Data and code availability

Raw sequences generated in this study are available in the NCBI SRA database under the BioProject accession PRJNA773605, and the China National Genomics Data Center (https://ngdc.cncb.ac.cn) under the BioProject accession PRJCA011616. Other data supporting the findings of this article are available in the supplemental information.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

J.-Q.C. and Z.-Q.S. designed the study. Z.Z. and Y.L. performed the experiments. Z.Z. analyzed the data and wrote the paper, with input from Y.L., X.-Y.F. and X.-M.J. Z.Z. and S.-X.L. constructed the database. J.-Q.C. and Z.-Q.S. revised the paper.

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