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Prediction and Analysis of miRNA Targets in Poplar in Response to the Infection of *Lonsdalea quercina* subsp. *Populi*

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Abstract Euramerican poplar bacterial canker disease caused by *Lonsdalea quercina* subsp. *Populi* is one of the stem diseases and seriously harmful to the growth and survival of poplar. MiRNAs play crucial roles in the regulation of the stress response of plants. To identify target genes of miRNAs responsive to the infection of this pathogen and provide candidate genes to the molecular breeding of forest disease resistance, the mature sequences of poplar miRNAs in miRBase database were used as probes to in silico predict the differentially expressed targets in poplar 'Zhonglin 46' inoculated with bacterial canker by using psRNATarget in this investigation. A total of 566 target genes of 276 miRNAs belonging to 127 miRNA families were screened, and these targets were found to be involved in multiple pathways such as plant pathogen interaction, plant hormone signaling, and phenylpropanoid biosynthesis, suggesting that these miRNAs were involved in the regulation of the response to *L. quercina* infection. Combined with GO analysis, KEGG pathway enrichment and gene function annotation of target genes, it was showed that miRNAs including miR482, miR6459, miR7812 and miR7835 were predicted to target genes encoding RPS2, NBS-LRR, AUX1, CCR, and Ca^{2+} transmembrane transport protein genes, which were involved in the resistance regulation of poplar to *L. quercina* infection. Six of the nine differentially expressed genes targeted by miR7835 encode the CCR enzyme in the phenylpropanol biosynthesis pathway, which might be involved in the defense response of poplars by affecting the synthesis of lignin. The miRNA:mRNA regulatory gene pairs found in this investigation enlarged the gene resources of molecular breeding of forest disease resistance, and provided new clues for the research of the molecular regulatory mechanism of disease resistance in trees.

Keywords Poplar; miRNA; Target gene; *Lonsdalea quercina* subsp. *Populi*

MicroRNA (miRNA) is a kind of small non-coding RNA with a length of about 22 nt, which widely exists in eukaryotes. It recognizes target genes by RNA-induced silencing complex RISC and regulates the expression of target genes by cutting target mRNA or inhibiting its translation. With the deepening of research, a large number of studies have shown that miRNA plays an important regulatory role in many aspects such as plant growth and development (Lian et al., 2018), cell differentiation (Pierre-Jerome et al., 2018), signal transduction (Sun et al., 2018), physiological metabolism (Bulgakov and Avramenko, 2015), biotic and abiotic stress responses (Lucas et al., 2014; Bai et al., 2018) through post-transcriptional gene regulation. In recent years, the regulatory role of miRNA in plant stress resistance and disease resistance has attracted wide attention.

It has been found that miRNA can regulate plant disease resistance directly or indirectly by targeting multiple genes. Many miRNA families such as miR472, miR482 and miR2118 affect the resistance of poplar, cotton, potato and tomato plants by targeting NBS-LRR resistance genes (Shivaprasad et al., 2012; Zhu et al., 2013; Yang et al., 2015a; Su et al., 2018). While miRNA interacts with NBS-LRR genes, the diversity of target genes also accelerates the evolution of miRNA (Zhang et al., 2016b). In addition to directly regulating the expression of disease resistance genes, miRNAs can also regulate many other genes, indirectly affecting plant sensitivity to pathogens. miR396 family regulates rice yield by inhibiting transcription factor (OsGRF) family genes in rice and

affects its blast resistance. Silencing miR396 can effectively improve rice yield and reduce its sensitivity to blast (Chandran et al., 2019), while miR319 can promote rice ragged stunt virus (RRSV) infection by inhibiting salicylic acid-mediated disease resistance in rice (Zhang et al., 2016a).

Plants form a variety of resistance mechanisms against pathogen infection, such as physical barriers formed by wax, cutin, lignin in cell walls, or chemical defenses formed by some phenolic and terpenoid secondary metabolites secreted by plant cells (Ding and Yang, 2016). In addition, plants also evolved two defense lines against pathogens, namely PAMP-triggered immunity (PTI) and effector-triggered-immunity (ETI). In the process of plant ETI, plant disease resistance protein (R protein) first recognizes the effect factors secreted by pathogens, and then triggers phosphorylation reactions in cells around the recognition site. At the same time, the ion flux in cells increases significantly, especially the change of Ca^{2+} is the most obvious, which is one of the early signals of plant disease resistance. In addition, salicylic acid (SA), as a further response signal to systemic disease resistance in plants, can rapidly induce reactive oxygen species (ROS) and nitric oxide (NO), thereby triggering hypersensitive response (HR) in plants, resulting in local programmed cell death (PCD) (Holt et al., 2000). It is now recognized that SA signaling inhibits biotrophic pathogens, while jasmonic acid (JAs) and ethylene (ET) pathways synergistically inhibit necrotrophic pathogens and antagonize SA signaling. However, there are a few exceptions where these hormones work together to protect against the invasion of external pathogens (Yang et al., 2015b). Singh et al. (2019) suggested that the methyl jasminate (MJ) in JA signal pathway could induce resistance to *Septoria tritici* by enhancing phenylpropanoid activity. NBS-LRR genes are the largest class of known R genes and widely exist in many plants (Tamura and Tachida, 2011). In poplars, these disease resistance genes are larger and more diverse. Kohler et al. (2008) identified 402 resistance genes in *Populus trichocarpa* genome by sequence alignment, of which 317 genes belonged to NBS-LRR type.

Euramerican poplar bacterial canker disease is one of the stem diseases caused by *Lonsdalea quercina* subsp. *Populi*, which was first found in Puyang City, Henan Province in 2006. Its symptoms were as follows: the bark of poplar stem cracked, and white acidic liquid seeped out. With the development of the disease, the bark was ulcerated and accompanied by foamy exudate, which could cause tree death in severe cases. At present, there is no lasting and efficient chemical control method for this disease (Li et al., 2014). To prevent the spread of bacterial canker and endanger forestry production, the breeding of resistant tree species is particularly important. On the basis of previous studies on poplar anti-ulcer genes and miRNAs, this study further identified the target genes of disease resistance-related miRNAs, analyzed their expression patterns, and annotated the function of target genes and their metabolic pathways by bioinformatics analysis, to provide some theoretical clues for the study of gene regulation network and molecular mechanism of disease resistance in tree disease resistance, and provide new candidate genes for tree disease resistance molecular breeding.

1 Results and Analysis

1.1 Prediction of miRNA candidate target genes

In this study, 566 differentially expressed target genes (DETGs) corresponding to 276 miRNAs from 127 miRNA families were predicted by psRNATarget online software. The results showed that a total of 16 miRNA families with 9 or more target genes were predicted, indicating that these miRNA family members may play a broad role in poplar resistance to canker infection, and are important candidate molecules for the study of poplar gene resistance regulation (Figure 1). Among them, miR482 is the most predicted target gene family, a total of 23 differentially expressed target genes were predicted, indicating that the regulatory function of miR482 family in plant disease resistance is conservative, and it should play an important regulatory role in poplar resistance to bacterial canker.

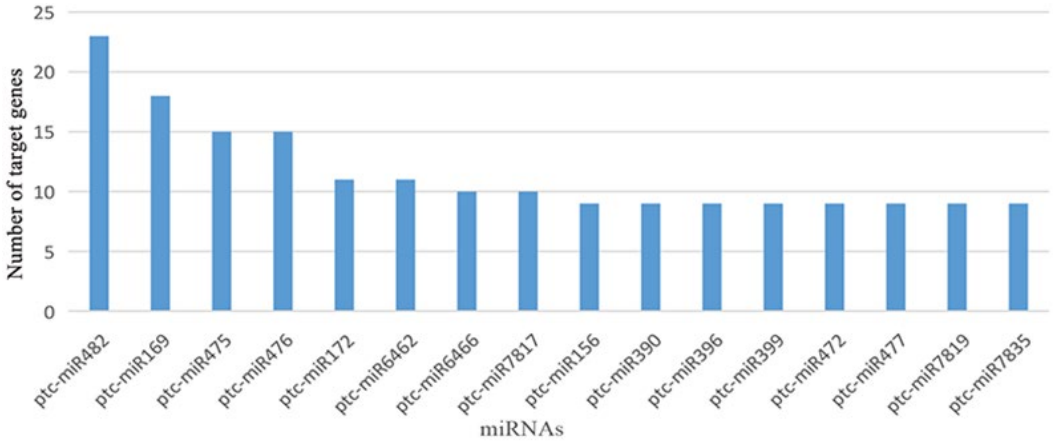

Figure 1 Distribution of the predicted target genes with the numbers no less than 9

1.2 Functional annotation of differentially expressed target genes

Based on the sequence homology, GO annotation analysis showed that 353 genes (62.37%) from the 566 differentially expressed target genes were annotated to 476 GO terms, among which 36 GO terms were classified by cellular component, 255 GO terms were classified by biological process, and 185 GO terms were classified by molecular function. Among them, the 4 GO terms of signal transducer activity, signal receptor activity, receptor activity and molecular transducer activity enriched significantly (p<0.05), which belonged to the molecular function classification (Figure 2), indicating that signal transduction plays an important regulatory role in the process of poplar disease resistance. In these 4 GO terms, a total of 8 genes (Potri.001G374600, Potri.001G374700, Potri.001G374800, Potri.004G052600, Potri.008G137900, Potri.009G168300, Potri.010G102900, Potri.010G145900) were significantly enriched, indicating that these genes may respond to the infection of *Lonsdalea quercina* subsp. *Populi* by participating in signal and molecular transduction.

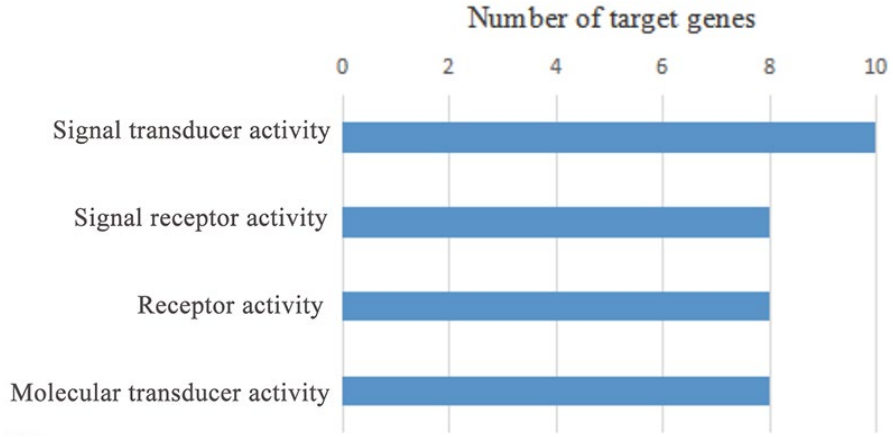

Figure 2 Significantly enriched GO terms of the target genes with differential expression

1.3 Analysis of metabolic pathways of differentially expressed target genes

In the KEGG enrichment analysis, a total of 129 differentially expressed target genes were enriched in 84 metabolic pathways, and 6 pathways enriched in more than 10 target genes. Only the *P* and *Q* values of metabolic pathways, biosynthesis of secondary metabolites and plant hormone signal transduction pathways were less than 0.05, indicating that they were significantly enriched pathways (Table 1).

In the significantly enriched pathway, only the plant hormone signal transduction pathway obtained protein annotation (Table 2). Most auxin-related proteins were enriched in this pathway. Both miR6459b and miR7812 were predicted to target auxin input vector AUX1 encoding genes. The target gene of miR6444 encoded SAUR family proteins, which could respond to auxin signals. The expression levels of the above target genes were

down-regulated after infection. The target gene of miR160 encodes cytokinin receptor protein and its expression is also down-regulated. It is speculated that the above hormone-related proteins may play a synergistic role in disease resistance regulation. In addition, MYC and TGA transcription factors are also enriched, which may respond to plant disease resistance and immunity by regulating the expression of downstream genes.

Table 1 KEGG pathway enrichment of the target genes with differential expression

KEGG pathway	Gene number (percentage)	Predicted protein
Metabolic pathways	69(53.49%)	No annotation
Biosynthesis of secondary metabolites	56(43.41%)	No annotation
Plant hormone signal transduction	17(13.18%)	AUX1, CRE1, SAUR, TIFY, PP2C, BZR1/2, COI1, PIF3, MYC2, NPR1, TGA, PR1
Carbon metabolism	14(10.85%)	HK, G6PD, PGD, ALDO, glpX-SEBP, GLDC, SHMT, aceB, ENO, aceE, ACAT, MDH1, OGDH
Plant-pathogen interaction	13(10.08%)	CDPK, CML, FLS2, PR1, RPS2, MYB, SLC45A3
Phenylpropanoid biosynthesis	10(7.75%)	COMT, F5H, CCOAMT, CCR, Prx2540

Note: * represent significantly enriched KEGG pathway; The values in brackets represent the proportion of the number of target genes enriched in the metabolic pathway in all the target genes enriched

Table 2 Target genes involved in plant hormone signal transduction

Target gene	Expression	miRNA	Predicted protein	Function
Potri.012G002700	↑	miR6445a,b	Protein phosphatase 2C	Protein serine/threonine phosphatase activity
Potri.014G111400	↓	miR399i	Phytochrome-interacting factor 3	Protein dimerization activity; protein binding
Potri.T131500	↑	miR6444	Pathogenesis-related protein 1	Response to jasmonic acid stimulus; systemic acquired resistance
Potri.008G137900	↓	miR160a,b-5p,c-5p,d	Histidine kinase 2/3/4 (cytokinin receptor)	Phosphorelay sensor kinase activity; cytokinin receptor activity
Potri.010G102900	↓	miR160a,b-5p,c-5p,d	Histidine kinase 2/3/4 (cytokinin receptor)	Phosphorelay sensor kinase Activity; cytokinin receptor activity
Potri.008G133400	↓	miR169n-3p	TIFY domain	Jasmonate ZIM domain-containing protein
Potri.002G176900	↓	miR473a-3p	Transcription factor MYC2	Protein dimerization activity
Potri.009G126000	↓	miR6444	SAUR family protein	Response to auxin
Potri.001G083500	↑	miR6459a-3p	Transcription factor MYC2	Protein dimerization activity
Potri.008G066400	↓	miR6459b	Auxin influx carrier AUX1 LAX family	Auxin transporter protein
Potri.005G206100	↓	miR6462e,f	Regulatory protein NPR1	Protein binding
Potri.005G082000	↓	miR6466-5p	Transcription factor TGA	Transcription factor activity
Potri.008G064400	↓	miR6470	Coronatine-insensitive protein 1	Protein binding
Potri.005G174000	↓	miR7812	Auxin influx carrier AUX1 LAX family	Auxin transporter-like protein
Potri.002G087000	↓	miR7812	Auxin influx carrier AUX1 LAX family	Auxin transporter-like protein 2-related
Potri.010G191000	↓	miR7812	Auxin influx carrier AUX1 LAX family	Auxin transporter protein
Potri.002G133700	↓	miR7836, 7837	Brassinosteroid resistant 1_2	BES1/BZR1 plant transcription factor

Plant-pathogen interaction pathway and plant hormone signaling pathway were enriched in the target gene of miR6444 Potri.T131500—encoding disease-related protein 1 involved in response to jasmonic acid stimulation, indicating that there may be some functional and material interactions between the two pathways. Most of the target genes enriched in plant-pathogen interaction pathways are directly related to disease resistance (Table 3). Like RPS2 has a typical LRR domain, which belongs to R protein. CDPK is a Ca^{2+}-related kinase protein. Both FLS2 and CML have Ca^{2+} binding function, which may be involved in poplar disease resistance by responding to

Ca^{2+} signal. MYB transcription factor enriched most in the pathway, which has a DNA binding domain, and may participate in the regulation of poplar disease resistance by interacting with some DNA.

Many enzyme-coding genes were enriched in phenylpropanoid biosynthesis pathway (Table 4). Among them, cinnamoyl-CoA reductase (CCR) had the highest enrichment degree, and all were targeted by miR7835. It is worth noting that 6 genes (Potri.001G045000, Potri.001G045100, Potri.001G045500, Potri.001G045800, Potri.001G046100, Potri.001G046400) of the 9 differentially expressed target genes of miR7835 encode CCR, which can be involved in steroid biosynthesis, and its function in disease resistance is worthy of further study.

Table 3 Target genes involved in plant-pathogen interaction

Target gene	Expression	miRNA	Predicted protein	Function
Potri.007G112400	↓	miR6479	Calcium-dependent protein kinase	Protein tyrosine kinase activity
Potri.016G066700	↑	miR7815	Calcium-dependent protein kinase	Protein tyrosine kinase activity; catalytic activity; protein phosphorylation
Potri.016G043600	↑	miR156k	Calcium-binding protein	EF hand family protein
Potri.004G065400	↑	miR478e	Flagellin-sensitive 2	Calcium ion binding; protein serine /threonine kinase activity
Potri.T131500	↑	miR6444	Pathogenesis-related protein 1	Response to jasmonic acid stimulus; systemic acquired resistance
Potri.001G428100	↓	miR482a.2	Disease resistance protein RPS2	NB-ARC domain; leucine-rich repeat-containing protein
Potri.011G124000	↓	miR482b-3p	Disease resistance protein RPS2	NB-ARC domain; leucine-rich repeat-containing protein
Potri.011G127200	↑	miR395a	Disease resistance protein RPS2	NB-ARC domain; leucine-rich repeat-containing protein
Potri.005G096600	↑	miR6449	Transcription factor MYB	MYB-like DNA-binding domain
Potri.006G221800	↓	miR6470	Transcription factor MYB	MYB-like DNA-binding domain
Potri.015G046200	↑	miR478e	Transcription factor MYB	MYB-like DNA-binding domain
Potri.017G130300	↑	miR6439a	Transcription factor MYB	MYB-like DNA-binding domain
Potri.018G032200	↑	miR160e-3p	Solute carrier family 45, member 3	Catalytic activity; fatty acid biosynthetic process

Table 4 Target genes involved in phenylpropanoid biosynthesis

Target gene	Expression	miRNA	Predicted protein	Function
Potri.015G003600	↓	miR6427-3p	Prx2540 peroxidase	Peroxidase activity
Potri.005G117500	↓	miR6443	Ferulate-5-hydroxylase	Oxidoreductase activity
Potri.007G016400	↓	miR6462e,f	Ferulate-5-hydroxylase	Oxidoreductase activity
Potri.009G099800	↑	miR6475	Caffeoyl-CoA o-methyltransferase	O-methyltransferase activity
Potri.012G006800	↑	miR7814	Prx2540 peroxidase	Peroxidase activity
Potri.014G106600	↑	miR7817a	Caffeic acid 3-o-methyltransferase	Methyltransferase activity
Potri.001G045100	↑	miR7835	Cinnamoyl-CoA reductase	3-beta-hydroxy-delta5-steroid dehydrogenase activity; steroid biosynthetic process
Potri.001G046100	↑	miR7835	Cinnamoyl-CoA reductase	3-beta-hydroxy-delta5-steroid dehydrogenase activity; steroid biosynthetic process
Potri.001G046400	↑	miR7835	Cinnamoyl-CoA reductase	Catalytic activity; 3-beta-hydroxy-delta5-steroid dehydrogenase activity; steroid biosynthetic process
Potri.001G045500	↑	miR7835	Cinnamoyl-CoA reductase	Catalytic activity; 3-beta-hydroxy-delta5-steroid dehydrogenase activity; steroid biosynthetic process

1.4 Conjoint analysis of differentially expressed target genes and miRNAs

The expression changes of miRNA target genes predicted in this study were compared with the expression of miRNA in poplar that responded to canker in our previous study (Table 5; Table 6), of which 11 miRNAs showed an opposite trend with their target genes (Table 6). miR390, miR476 and miR482 predicted target genes with LRR structure (Potri.006G051700, Potri.002G260100, Potri.T105500), and miR390 also targeted gene Potri.013 G064300 encoding serine/threonine protein kinase. The above genes have resistance gene characteristics, which may be closely related to the response to pathogen stress. In addition to miR390, there are many miRNAs that target protein kinase genes and have protein binding function, which may play a role in poplar disease resistance through protein interaction. The target gene Potri.001G020600 of miR396 encodes cation transport ATPase, which is responsible for the transmembrane transport of Ca^{2+} and may be involved in the transmission of disease resistance signals.

To further confirm the targeting relationship between miRNA and its target genes, the base complementary pairing of some miRNA and its target genes was compared. Complementary pairing of miRNA and its target genes showed that miRNA and its target genes were highly complementary, especially in the seed sequence region, only individual base mismatch and a small amount of G:U wobble pairing (Table 7).

2 Discussion

Plant disease is an important obstacle to agricultural and forestry production. Crop yield reduction and forest decline caused by plant diseases have led to serious economic losses. Therefore, plant disease resistance breeding is very important. R gene is recognized as a disease-resistant gene, and majority of R genes contain leucine-rich repeat (LRR) domains. Since the LRR domain can interact directly or indirectly with the AVR gene products of pathogens, it is considered to play a major role in identifying pathogens (Jia et al., 2000). In this experiment, the predicted target genes of miR390, miR476 and miR482 all had LRR structure, indicating that these three miRNAs may be involved in the regulation of poplar response to bacterial canker infection. The specific functions of these important disease-resistant genes and miRNAs in poplar disease resistance need to be further studied in subsequent transgenic experiments. Interesting that the expression levels of the above target genes were down-regulated when poplars were infected by pathogens, while the miRNA targeting them was up-regulated. Previous studies have shown that the expression of miR1450 target gene (LRR transmembrane protein gene) in poplar was significantly lower at 7 d after infection than at 5 d (Zhao et al., 2012b), indicating that the above genes may play a role in the defensive response of poplar in the early stage of infection.

Auxins are associated with plant growth and development, but studies have shown that auxins can also respond to plant disease resistance and antagonize SA signaling pathways (Pieterse et al., 2009). Studies have confirmed that miRNA can regulate plant auxin signal pathway and play an important role in disease resistance. Liao et al. (2014, Chinese Journal of Cell Biology, 36(11): 1506-1513) suggested that the miR393 was involved in auxin signal transduction by targeting *TIR1*, *AFB2* and *AFB3* genes in *Populus tomentosa* under pathogen stress, thus responding to pathogen invasion. In this experiment, we found that the other two miRNAs in poplar were involved in the regulation of auxin signaling pathway. The target genes of miR6459b (Potri.008G066400) and miR7812 (Potri.005G174000, Potri.002G087000, Potri.010G191000) encoded auxin input vector AUX1, and these target genes were significantly enriched in the plant hormone signal transduction pathway. Their expression levels showed a significantly down-regulated trend after inoculation of poplar ‘Zhonglin 46’. It was speculated that miR6459 and miR7812 might regulate plant disease resistance by controlling auxin transfer and interfering with salicylic acid signal transduction.

Table 5 Comparison of differentially expressed genes and miRNAs

miRNAs	Expression	Fold change	Target gene	Expression
miR1446a	↑	2.66	Potri.013G010800	↑
			Potri.002G135100	↑
miR160a	↓	0.76	Potri.008G137900	↓
			Potri.010G102900	↓
miR164a	↑	2.36	Potri.010G152000	↓
miR168a-5p	↑	3.80	Potri.016G128500	↓
			Potri.008G144700	↑
miR169s	↑	1.99	Potri.009G104100	↑
			Potri.009G104000	↑
			Potri.011G140000	↓
			Potri.009G122100	↑
			Potri.015G131100	↑
miR171a-3p	↑	1.38	Potri.015G096200	↓
			Potri.007G029200	↓
miR171f	↑	2.01	Potri.008G188600	↑
miR2111a	↓	0.25	Potri.018G150400	↓
miR390a	↑	2.10	Potri.002G258000	↑
			Potri.001G325100	↑
			Potri.013G064300	↓
			Potri.006G051700	↓
			Potri.010G254700	↓
miR393a-5p	↑	8.27	Potri.004G038500	↑
			Potri.004G089100	↓
miR396a	↓	0.82	Potri.001G020600	↑
			Potri.015G092000	↓
miR397a	↓	0.60	Potri.004G015000	↓
			Potri.009G074800	↓
miR472a	↓	0.88	Potri.017G121500	↓
			Potri.001G442800	↑
miR472b	↓	0.98	Potri.003G200100	↓
			Potri.003G200200	↓
			Potri.003G034200	↓
			Potri.003G199100	↓
			Potri.003G199400	↓
			Potri.003G199600	↓
			Potri.018G100900	↓
miR476a	↑	3.05	Potri.001G374800	↑
			Potri.001G374700	↑
			Potri.001G374600	↑
			Potri.006G100500	↑
			Potri.001G343400	↑
			Potri.019G063400	↓
			Potri.017G108100	↓
			Potri.002G260100	↓
miR482a.2	↓	0.67	Potri.001G428100	↓
			Potri.006G191800	↓
miR482c-3p	↑	2.49	Potri.005G136200	↓
			Potri.003G097100	↓
			Potri.T105500	↓
			Potri.018G151300	↓
			Potri.T004100	↓
miR530a	↑	1.04	Potri.008G116500	↑
			Potri.001G347400	↑
			Potri.002G178500	↓
			Potri.002G113300	↓

Table 6 The target genes and miRNAs with the opposite trend in expression change

MiRNAs	Expression	Target genes	Expression	Encoded protein	Function
miR164a	↑	Potri.010G152000	↓	Polygalacturonase / pectinase	Pectate lyase suerfamily protein
miR168a-5p	↑	Potri.016G128500	↓	Solute carrier family 40 (iron-regulated transporter) member 1	Iron ion transmembrane transport
miR169s	↑	Potri.011G140000	↓	Kinesin motor protein	Microtubule motor activity
miR171a-3p	↑	Potri.015G096200	↓	Programmed cell death protein 2-related	Zinc finger mynd 2; protein binding
		Potri.007G029200	↓	Scarecrow-like protein 15	GRASdomain family
miR390a	↑	Potri.013G064300	↓	Serine/threonine protein kinase	Protein kinase activity; protein phosphorylation
		Potri.006G051700	↓	Leucine-rich repeat; Transmembrane protein; Kinase	Protein kinase activity; protein phosphorylation
		Potri.010G254700	↓	Serine hydroxymethyltransferase	Transferase activity; catalytic activity
miR393a-5p	↑	Potri.004G089100	↓	Threonine specific protein kinase	Protein kinase activity; protein phosphorylation
miR396a	↓	Potri.001G020600	↑	Cation transporting atpase	Calcium ion transmembrane transport
miR472a	↓	Potri.001G442800	↑	Maltase-glucoamylase	Carbohydrate metabolic process
miR476a	↑	Potri.019G063400	↓	Fad binding domain; berberine	Oxidation-reduction process
		Potri.017G108100	↓	Pentatricopeptide repeat-containing protein	Protein binding
		Potri.002G260100	↓	Protein tyrosine kinase, pkinase_tyr; leucine rich repeat, LRR_8	Protein kinase activity
miR482c-3p	↑	Potri.005G136200	↓	Enoyl-(acyl carrier protein) reductase	Oxidoreductase activity
		Potri.003G097100	↓	Cellulose synthase-like D1 protein	Zinc/ring finger domain
		Potri.T105500	↓	Leucine rich repeat-containing protein	ADP binding; disease resistance protein signature
		Potri.018G151300	↓	Transcriptional regulator	Nucleotide binding
		Potri.T004100	↓	Leucine rich repeat (LRR_8)	Protein binding
miR530a	↑	Potri.002G178500	↓	Protein IQ-domain 17-related	Protein binding
		Potri.002G113300	↓	Kn1-like protein	Regulation of transcription

Table 7 The complementary sites of miRNAs and target genes

Gene name	Sequence alignment
ptc-miR390a	A A G C U C A G G A G G G A U A G C G C C
	| | | | | | | | | | | | * | | | * | | | |
Potri.006G051700	U U C G A G U C G U C C U U A U U G A G G
ptc-miR476a	U A G U A A U C C U U C U U U G C A A A G
	| | | | | | * | | | | | * | | | | | | |
Potri.002G260100	A U C A U U G G A A A G G A A C C U U U A
ptc-miR482c-3p	U C U U U C C G A G U C C U C C C A U A C C
	| | | * | | | | | * | | * | | | | | | | |
Potri.T105500	A A A A G G G C U C G G G G G G G U A A G G
ptc-miR482c-3p	U C U U U C C G A G U C C U C C C A U A C C
	| | | | | | | | | | | | | | | | | | | |
Potri.T004100	A G A A A G G G U G A G G C G G G U A G G G
ptc-miR396a	U U C C A C A G C U U U C U U G A A C U G
	| | | | | | * | | | * | | | * | | |
Potri.001G020600	A C G G U G U U G A A G G A A U U U G U G

Note: Vertical lines represent Watson-Crick pairing; "*" represents G:U wobble pairing

miR482 family has been proved to be able to regulate plant disease resistance in cotton, tomato, poplar and other plants (Zhao et al., 2012a; Zhu et al., 2013; De Vries et al., 2018). In this experiment, a total of 23 differentially expressed target genes in the miR482 family were predicted. Among them, the target gene Potri.001G428100 of miR482a.2 and the target gene Potri.001G428100 of miR482b-3p encoded the resistance protein RPS2 and were enriched in the plant-pathogen interaction pathway. The target gene Potri.015G043600 of miR482d-3p was a TIR-NBS-LRR resistance gene, and miR482a.2, miR482c-3p and miR482d-3p predicted the target gene with LRR structure. Thus, the regulation of miR482 family members on poplar disease resistance may be conservative. In contrast to the predicted target gene Potri.001G020600, miR396a encoded cation transport ATPase and participated in Ca^{2+} transmembrane transport. The change of Ca^{2+} concentration is one of the early signals of plant-pathogen interaction, and its transient or continuous increase is often accompanied by PTI or ETI in plants (Aldon et al., 2018). Previous studies have shown that miR396 affects the disease resistance of Arabidopsis, rice and tomato (Chen et al., 2017; Soto-Suárez et al., 2017; Chandran et al., 2019). In this experiment, the differential expression of miRNA and its target genes indicated that miR396 could also respond to poplar bacterial canker infection. Previous studies on miR7835 were rare. In this study, a total of nine differentially expressed target genes were predicted, of which six genes encoded CCR and four were enriched in phenylpropanoid biosynthetic pathway. The target genes encoded by these CCRs were all located on chromosome 1 of poplar, showing an upward trend after infection. Previous studies have shown that CCR is an important regulatory point of carbon flux in lignin synthesis. In phenylpropanoid biosynthesis pathway, CCR can catalyze the conversion of cinnamoyl-CoA into cinnamaldehyde, and also convert feruloyl-CoA into coniferylaldehyde, which is an essential catalyst for lignin synthesis. The differential expression of CCR will affect the lignin content of plants and lead to changes in lignin composition (Chao et al., 2017). Lee et al. (2019) found that 6-week-old Arabidopsis plants inoculated with nontoxic pathogens showed significantly increased lignification, indicating that lignin can respond to pathogen stress. These results showed that the above genes may jointly promote lignin synthesis, thereby increasing poplar resistance to pathogen infection.

3 Materials and Methods

3.1 Expression analysis of target genes in poplar infected by pathogen

The transcriptome sequencing data (Accession No.: SRP057770) of inoculated and uninoculated poplar 'Zhonglin 46' after 6 d of bacterial canker disease were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/), and the sequence was compared with the reference genome of *Populus trichocarpa* (http://www.phytozome.net/poplar.php, Phytozome v12.1, Poplar v3.0) by SOAPaligner/soap2 to obtain the gene number and functional annotation (Li et al., 2009). FPKM (fragments per kilobase of transcript per Million mapped reads) was used to calculate gene expression levels. The formula is as follows:

FPKM=mapped fragment of transcript/(total count of mapped fragments×length of transcript)

The genes that the expression changes were more than two times (up or down) and FDR≤0.001 were selected as differential expression genes (Hou et al., 2016).

3.2 Prediction of miRNA target genes in poplar

The mature sequence of poplar miRNA was obtained from microRNA database website miRBase (http://www.mirbase.org/, release 22) and used as a probe. The target genes were predicted from the differentially expressed genes by using the online prediction software psRNATarget for plant small RNA target genes. All parameters were used by default values (Dai and Zhao, 2011).

3.3 GO functional annotation of differentially expressed target genes

GO (Gene ontology) annotation analysis was performed on the obtained differentially expressed target genes. The functional annotations of genes were obtained by Blast2GO (http://www.blast2go.org/) comparison, and the corresponding GO annotation information was obtained according to the corresponding relationship in the database. $P<0.05$ was used to determine the enrichment of differentially expressed target genes in GO

classification, and the enrichment of differentially expressed target genes in biological process, molecular function and cellular component was statistically analyzed (Armstrong, 2014; Hou et al., 2016).

3.4 KEGG metabolic pathway analysis of differentially expressed target genes

The KEGG metabolic pathway function of the predicted differentially expressed target genes was analyzed. KAAS (KEGG Automatic Annotation Server) software was used to obtain the corresponding KO number using Blast information. $P<0.05$ and $Q\leq0.05$ were used as the standard of significant enrichment (Hou et al., 2016; Armstrong, 2014) to analyze the enrichment of target genes in different metabolic pathways of poplar after inoculation with bacterial canker.

3.5 Conjoint analysis of differentially expressed target genes and miRNAs

Based on our previous study on the differential expression of miRNAs in poplar infected by canker (Chen et al., 2012), combined with the predicted miRNA target gene expression changes in this study, we corresponded them one-to-one and found out the gene pairs in which the expression of miRNA and its target genes show the opposite trend for conjoint analysis.

Authors' contributions

XQH was the executor of this experiment, who completed the data analysis and wrote the first draft of paper. CFL participated in experimental design and material preparation. HXW and ZK participated in data analysis. WYW was the project designer and director, guiding experimental design, data analysis, writing and revision. All authors read and approved the final manuscript.

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