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Identification of tomato circular RNAs in response to *Botrytis cinerea*

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Abstract: *Botrytis cinerea* is one of the main pathogens that harm the tomato yield and cause huge economic losses worldwide. Studies of circRNAs in response to the stress caused by pathogens have received more and more attention in tomato and other model crops. In this study, four groups were treated with ZaoFen (ZF), CuiLi (CL) (susceptible and tolerant genotypes to *B. cinerea*, respectively), ZFBc, CLBc (48 hour response to a *B. cinerea* infection). A total of 918 circRNAs were identified, among which exonic circRNAs (70.70%) accounted for the majority of them, and 118 circRNAs (12.85%) were located in chr1. A total of 18 (1.96%) circRNAs were shared among the four libraries. A total of 6 circRNAs showed fold changes in the differential expression analysis between the time and cultivar control groups, and circRNA115, circRNA145 and circRNA223 repeatedly appeared in the different control treatments. Notably, the gene targeted by circRNA115 was an ethylene-forming enzyme. At the same time, we predicted the target genes of the six circRNAs obtained in the study, and a total of 319 miRNAs were predicted. This study contributes to the mechanism in response to *B. cinerea* stress in the tomato, and paves the way for the further study of circRNAs under tomato pathogen stress.

Keywords: *Solanum lycopersicum*; grey mould; circRNAs; ethylene-forming enzyme

Circular RNAs (circRNAs) are endogenous non-coding RNAs that have been observed in eukaryotic cells for decades (Zhou et al. 2018; Zhou et al. 2020). Although circRNAs have been intensively studied recently in humans and other animals (Zhou et al. 2018), we know little about their origin and roles in plants. Recently, the genome-wide identification of circRNAs in plants have been conducted in the stress responses of several plant species (Wang et al. 2016; Hong et al. 2020). For example, 371 circRNAs in soybeans and 27 circRNAs in rice have been found to be differentially expressed under low-phosphorus stress (Lv et al. 2020). Compared with normal calcium, calcium deficiency in different stages led to the different expression of 23 and 22 circRNAs among 730 circRNAs in Chinese cabbage (Wang et al. 2019). There have been some reports about circRNAs being involved in abiotic stress, but there are few reports about circRNAs being involved in biotic stress. Eighty-three (83) circRNAs were differentially expressed in tomatoes infected by yellow leaf curl virus (Wang et al. 2018). Sixty-eight (68) circRNAs were identified in tomatoes in response to a *Phytophthora infestans* infection by high-throughput sequencing (Hong et al. 2020).

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The tomato (*Solanum lycopersicum*) is one of the most important horticultural crops worldwide. Meanwhile, tomatoes can be used as a model plant in such agricultural research as fruit development and ripening, disease resistance, and biochemical pathways to synthesise important nutrients (Ding et al. 2020). Many commercial tomato cultivars are susceptible to *Botrytis cinerea* (Nambeesan et al. 2012). *B. cinerea* is a necrotrophic pathogen which can infect tomato tissues, such as leaves, stems, flowers, and fruits, then kill tomato cells and feed on the decayed tissues, resulting in over 30% yield losses and significant economic impacts during the growth stage and storage (Yao et al. 2011). Plant resistance against *B. cinerea* occurs by activating two kinds of pathogen response genes, the plant innate immune system consists of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), they play different roles in an infection by the different types of pathogens (Wang et al. 2018; Jones, Dangl 2006; Yuan et al. 2021). Generally, only the PTI of plants is activated to resist pathogens from necrosis (Yuan et al. 2021). The biological and molecular resistant mechanisms of tomato circRNAs against *B. cinerea* are far from being well understood.

In this study, we employed two representative tomato genotypes ZaoFen (ZF, susceptible to *B. cinerea*) and CuiLi (CL, resistant to *B. cinerea*), which are contrasting to each other in their response to a *B. cinerea* infection, to quantify the circRNAs in tomatoes, and to explore their potential functions in regulating the response to a *B. cinerea* infection. We performed genome-wide identification of circRNAs in tomato *B.cinerea* interaction by high-throughput RNA sequencing technology (RNA-seq). This study will serve as a pioneering one for future studies on the roles of circRNAs in plants under pathogen stress, especially necrotising pathogens stresses.

MATERIAL AND METHODS

Plant culture and sample collection. The seedlings of *Solanum lycopersicum* L. cv. ZaoFen (ZF) and CuiLi (CL) were grown in an artificial intelligence climate chamber under a photoperiod of 16 hours : 8 hours (light : dark) and a temperature of 25 °C/20 °C (day/night). When the fourth leaf was fully expanded, infection assays of *B. cinerea* were conducted, three independent biological replicates were performed for each treatment. The *B. cinerea* strain was provided by the Liaoning Academy of Agricultural Sciences, the grey mould inoculation method of Sun et al. (2021) was taken. The leaves were collected at 0 and 48 hpi, immediately frozen in liquid nitrogen, then stored at –80 °C for the following experiments.

Library construction, sequencing, and identification. The susceptible tomato cultivar of ZF and the resistant one of CL were selected for the circRNA library construction and RNA sequencing. The total RNAs were extracted from the tomato leaves by a Trizol kit (TaKaRa, Dalian, China). The RNA quality was examined using a 2100 Bioanalyzer (Agilent, CA, USA). The high-throughput RNA sequencing was performed using an Illumina Hiseq4000 of the LC-Bio Co., Ltd (Hangzhou, China). The raw data were purified as in Zhou et al. (2020)

Bioinformatic analysis and validation of the expressed circRNAs. The expression levels of the circRNAs were measured by spliced reads per billion mapping (srpbm). The differentially expressed (DE) circRNAs were identified by referring to those in Zhou et al. (2020). The enrichment analysis on the parent genes of the DE circRNAs was performed through the Gene ontology (GO) term enrichment and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Construction of the circRNA–miRNA–mRNA network. The software PsRobot was used to analyse the circRNA-targeted miRNA. The threshold for PsRobot software in this experiment was 5, under 5 the smaller the value of the result is, the higher the reliabity of the result is. The bioinformatic analysis was performed using OmicStudio tools at https://www.omicstudio.cn/tool.

RESULTS

Identification and characterisation of the circRNAs in tomatoes. A total of 89.15, 89.27, 96.49 and 94.56 million raw reads were generated from the ZF, ZFBc, CL, CLBc treatments, respectively. After further screening, 88.22 (ZF), 87.97 (ZFBc), 94.77 (CL), 93.16 (CLBc) million valid data reads were retained (Table 1). A total of 918 circRNAs were identified in the tomatoes in the four treatments (Figure 1A). The circRNAs from the ex-

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Table 1. Data summary of the circRNA sequencing in the tomatoes

Sample	Raw data (reads)	Valid data (reads)	Valid ratio (reads)	Q30 (%)	GC content (%)
ZF	89 150 362	88 226 592	98.96	94.02	42
ZFBc	89 274 168	87 973 818	98.54	95.18	42
CL	96 494 408	94 772 580	98.22	94.98	42
CLBc	94 555 054	93 161 214	98.53	94.98	42

ZF – ZaoFen; CL – CuiLi; GC – Guanine and Cytosine; ZFBc – Zaofen inoculted with *B.cinerea* 48 hours; CLBc – Cuili inoculted with *B.cinerea* 48 hours; Q30 – Quality sore 30

ons, intergenic regions, and introns accounted for 70.70%, 24.40%, and 4.90% of the all the circRNAs, respectively. The exonic circRNAs accounted for the majority of these reads, which was consistent with the study by Hong et al. (2020).

A total of 18 circRNAs were shared among the four libraries (ZF, CL, ZFBc, and CLBc), while 156, 139, 257 and 229 circRNAs were only present in the four groups of treatments, respectively (Figure 1B). 118 and 43 circRNAs were derived from chr1 and chr10, accounting for the largest and smallest proportions (12.85% and 4.68%, respectively) (Figure 1C). According to the length count range analysis, the most of the circRNAs were shorter than 10 kb. After 48 hpi, the number of ZF and CL circRNAs increased in all the length ranges (Figure 1D).

Comparison of the circRNA expression levels. 16 circRNAs were found to be significantly up-regulated in ZF VS ZFBc, while 8 circRNAs were significantly down-regulated (Figure 2A), and there were 3 circRNAs with a significant fold change in ZF_VS_ZFBc, namely circRNA219 (2.38 $\log_2$FC), circRNA220 (2.94$\log_2$FC) and the significantly down-regulated circRNA115 (–3.16$\log_2$FC) (Figure 2B) 30 circRNAs were significantly up-regulated in CL VS CLBc, while 10 were significantly down-regulated (Figure 2A). Under the treatment of 0 hpi and 48 hpi CL cultivars, the circRNA145 (3.07$\log_2$FC) and circRNA223 (–1.72$\log_2$FC) showed a fold change (Figure 2B).

In the ZF, VS and CL controls, it was found that 13 circRNAs were significantly up-regulated, while 7 were significantly down-regulated (Figure 2A), including circRNA145 (3.88$\log_2$FC) and circRNA223 (–3.17$\log_2$FC) (Figure 2B). In the ZFBc_VS_CLBc treatments, 28 circRNAs were significantly up-regulated, while 17 were significantly down-regulated (Figure 2A). Among them, circRNA473 (3.32$\log_2$FC) and circRNA20 (2.75$\log_2$FC) were significantly ameliorated, circRNA115 (–3.14$\log_2$FC) was restrained with a fold change (Figure 2B). Notably, it was found that circRNA115, circRNA145 and circRNA223 showed a significant fold change in the different treatment groups (Table 2).

CircRNA functional analysis. Additionally, the annotation of the circRNA115 parental gene was 1-aminocyclopropane-1-carboxylate oxidase (ACO), which was an ethylene-forming enzyme, which is related to signal functions such as stimulation of fruit ripening (Zhou et al. 2018). Moreover, the pathway remarkably enriched by KEGG was ko400270 (Cysteine and methionine metabolism, EC: 1.14.17.4). The annotation of the circRNA145 parental gene was a Transducin/WD40 repeat-like superfamily protein. Regrettably, the target gene of circRNA223 was not been mapped. Hence, the three circRNAs should be focused on in further studies.

Construction of the circRNA–miRNA–mRNA network. CircRNAs were shown to act as miRNA sponges and inhibit the miRNA activity through competition for the endogenous RNA (ceRNA) network. The target genes of circRNA115, circRNA145, circRNA185, circRNA187, circRNA20, circRNA219, circRNA220, circRNA223, and circRNA473 were predicted using the PsRobot soft-

Table 2. Data summary of the repeated differentially expressed circRNAs

circRNA	circRNA115	circRNA145	circRNA223
Different treatment groups	ZF_VS_ZFBc (–3.16$\log_2$FC)	CL_VS_CLBc (3.07$\log_2$FC)	ZFBc_VS_CLBc (–1.72$\log_2$FC)
	ZFBc_VS_CLBc (–3.14$\log_2$FC)	ZF_VS_CL (3.88$\log_2$FC)	ZF_VS_CL (–3.17$\log_2$FC)

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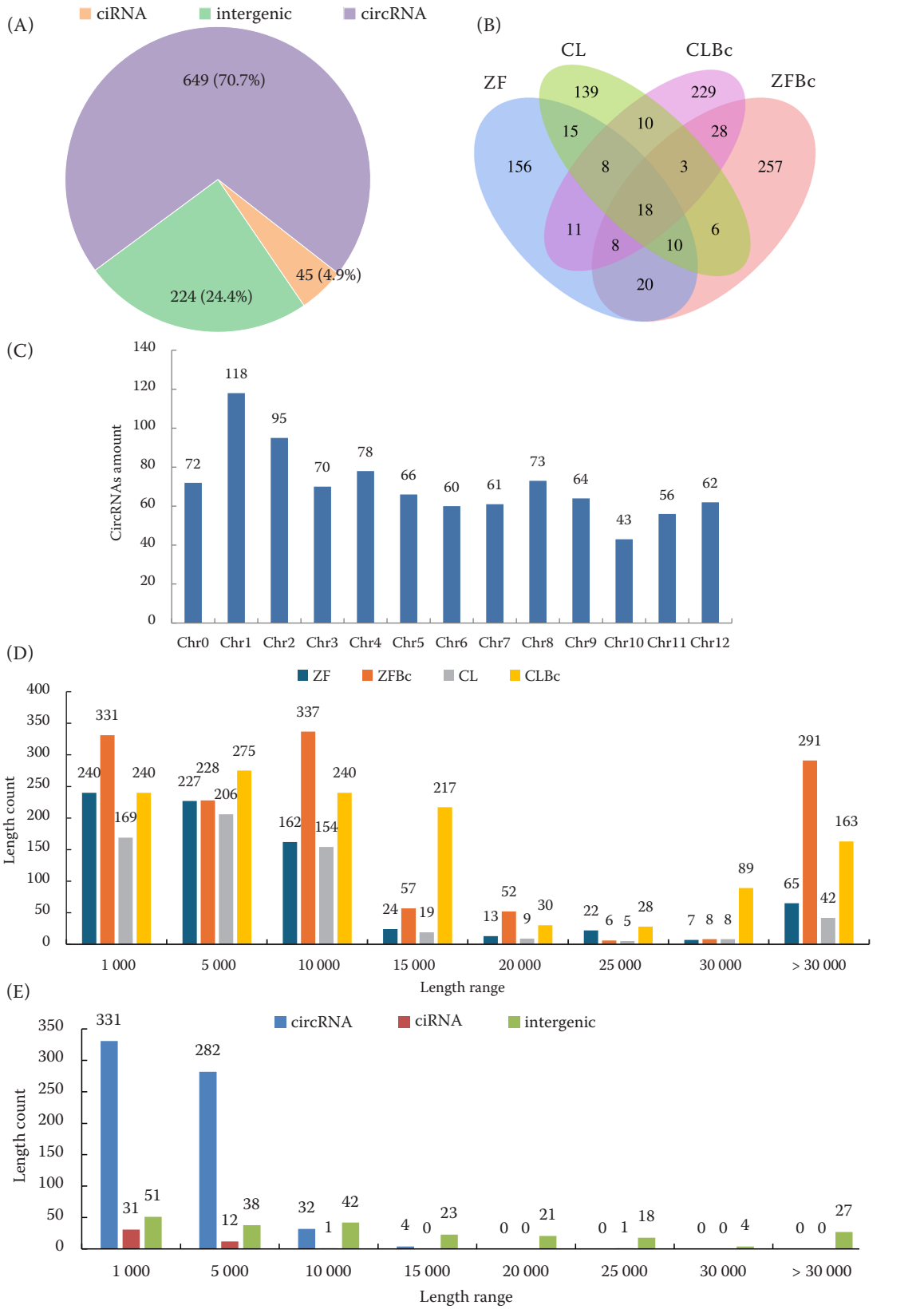

Figure 1. Identification and characterisation of the circRNAs in the tomatoes

(A) the amount and percentage of circRNAs originated from the exon, intron and intergenic region; (B) CircRNA amount identified in the ZF, CL, ZFBc and CLBc treatments. (C) the amount of circRNAs in each chromosome; (D) and (E) histogram of the amounts of circRNAs in the different length ranges; ZF, CL – for treatments explanation see Table 1

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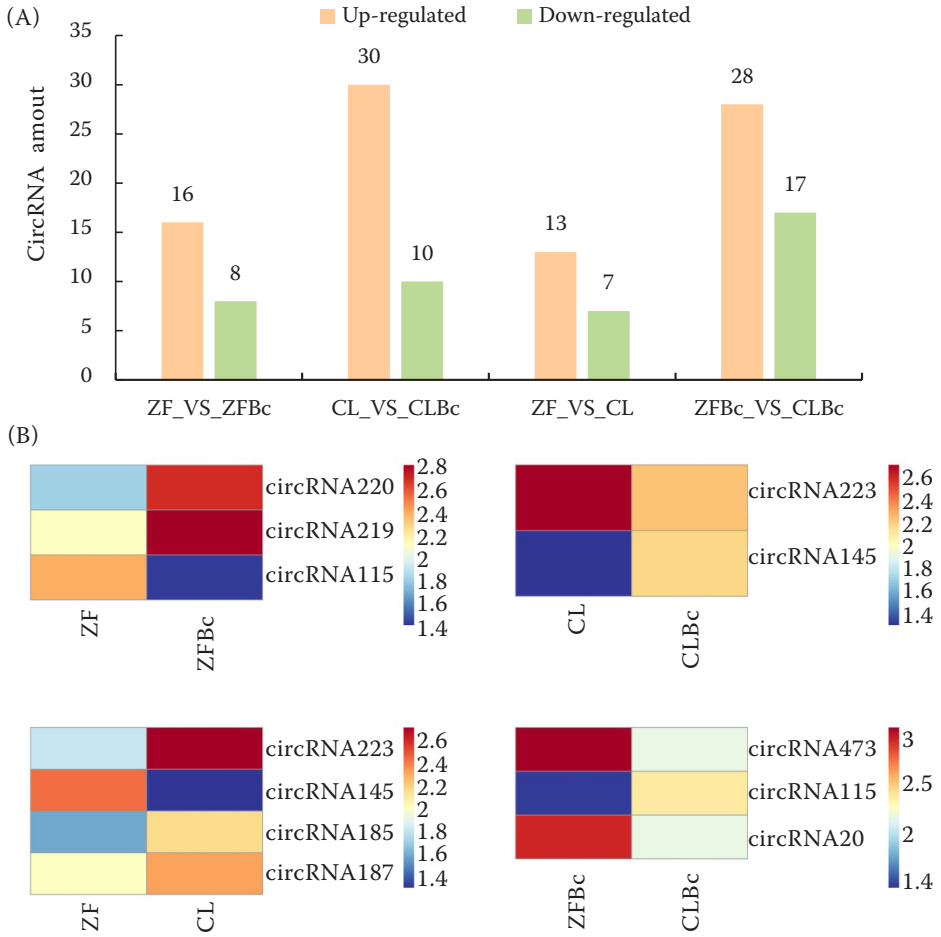

Figure 2. Comparison of the circRNA expression levels among the ZF_VS_ZFBc, CL_VS_CLBc, ZF_VS_CL, ZFBc_VS_CLBc treatments

(A) column chart of the differentially expressed circRNAs; (B) the heatmap showed the expression levels of the differentially expressed circRNAs; ZF, CL – for treatments explanation see Table 1

ware and then 5, 2, 10, 10, 3, 27, 27, 58, 319 targeted genes were predicted, respectively (Figure 3).

In reference to the target genes predicted by circRNA473 (sly-miR1916, sly-miR6022, sly-miR6024), the results of previous studies showed that microRNA1916 (miR1916) was a non-conserved miRNA response to various stresses in plants (*epidemic mould* or *B. cinerea*) (Chen et al. 2018). Sly-miR6022 regulates a tomato disease resistance gene against the leaf mould fungal pathogen (Li et al. 2016). Sly-miR6024 was responsible for reducing the tomato resistance to *B. cinerea* (Wang, 2019).

DISCUSSION

In tomatoes, circRNAs have been substantiated to be involved in fruit ripening regulation, *B. cinerea* responding processes and ethylene signalling pathways (Pirrung 1999; Dodds, Rathjen 2010; Wang et al. 2019). At present, it is generally agreed that plants recognise and resist pathogens mainly through two lines of innate immunity (Wang 2019).

In the course of four groups of treatments, 845 circRNAs were obtained. Among them, the circRNAs from exons, intergenic regions and introns accounted for 70.70%, 24.40%, and 4.90% of the circRNAs, respectively. However only 18 (1.96%) of the circRNAs were shared. Zhou et al. (2019) explored the potential functions of circRNAs in tomato seeds at high temperature. After high-throughput sequencing, entirely 4.164 circRNAs were identified, of which 980 (23.54%) circRNAs were shared between the control library and the high temperature library. Hong et al. (2020) explored the potential mechanism of action of circRNAs in tomatoes

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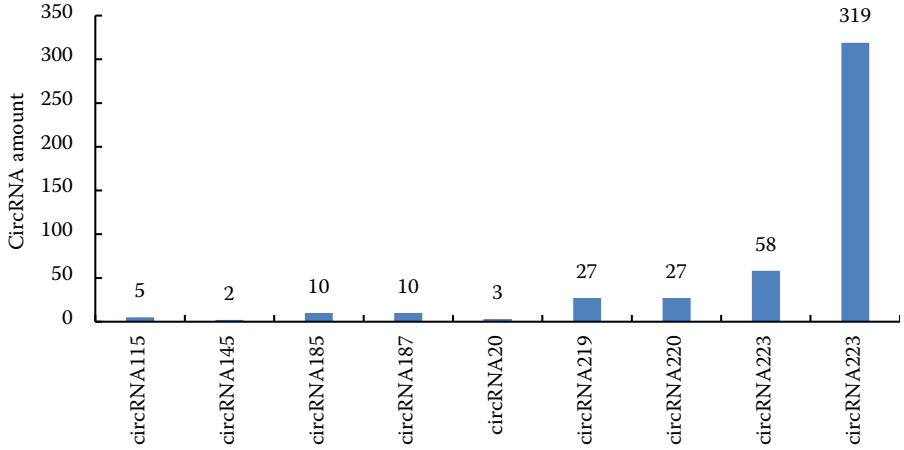

Figure 3. The predicted target genes for the special expression of circRNAs

under different treating time stresses of *P. infestans*, of which 9 (13.24%) circRNAs were shared. The differential expression of circRNA may play an important role in the tomato's susceptibility and resistance to the pathogenic stress of *B. cinerea*.

In four groups of different controls (ZF_VS_ZFBc, CL_VS_CLBc, ZF_VS_CL, ZFBc_VS_CLBc), 6 circRNAs were found with a fold change. Among them, circRNA115, circRNA223 and circRNA145 appeared repeatedly in the different controls. It can be preliminarily presumed that they play an important role in resistance to grey mould stress. Among them, the circRNA115 mapping target gene (ACO) is associated with ethylene-forming immunity (Thrower et al. 2001). Ethylene (ET) production is a hallmark of PTI.

Circular RNAs play a major role in plant immunity (Song et al. 2021), but, so far, little is known about the role of circRNA-miRNA-mRNA cascades in plant-pathogen interactions (Hong et al. 2020). Zuo et al. (2016) found 102 circRNAs to act as corresponding 24 miRNA sponges in tomatoes, and predicted 461 target genes by using the PsRobot software, of which, circRNA473 deals with 319 target genes. We focused on the target genes predicted by circRNA473 (sly-miR1916, sly-miR6022, sly-miR6024), which were previously reported to be related to disease stress. Hong et al. (2020) reported that circRNA45 and circRNA47 may act as miR477-3P sponges, which were involved in regulating the expression of the target resistance genes *SpRLK1/2*, and play a positive regulatory role in tomato immunity against *P. infestans*. The construction of the CircRNA-miRNA-mRNA network will be the focus of our next work.

In this study, it can be preliminarily speculated that circRNA115, circRNA223, and circRNA145 play an important role in resisting grey mould stress. In addition, the regulatory mechanism of 1-aminocyclopane-1-carboxylate oxidase involved in disease resistance and stress resistance also needs further in-depth research. Our results generated form the genome-wide identification of circRNAs in tomato seedlings, which especially enrich the number of circRNAs in plant response to a *B. cinerea* infection. It also provides a solid basis for the further in-depth exploration of the biological functions of circRNAs in tomato-pathogen interaction.

REFERENCES

Chen L., Meng J., He X., Zhang M., Luan Y. (2018): Sly-miR1916 targets multiple target genes and negatively regulates the immune response in tomato. Plant Cell & Environment, 42: 1393–1407.

Ding Q., Yang X., Pi Y., Li Z., Xue J., Chen H., Wu H. (2020): Genome-wide identification and expression analysis of extensin genes in tomato. Genomic, 112: 4348–4360.

Dodds P., Rathjen J. (2010): Plant immunity: towards an integrated view of plant-pathogen interactions. Nature Reviews Genetics, 11: 539–548.

Hong Y.H., Meng J., Zhang M., Luan YS. (2020): Identification of tomato circular RNAs responsive to *Phytophthora infestans*. Gene: 144652.

Jones J., Dangl J. (2006): The plant immune system. Nature, 444: 323–329.

Li W., Xu Y., Cai Z. (2016): Transcriptional and posttranscriptional regulation of the tomato leaf mould disease resistance gene Cf-9. Biochemical and Biophysical Research Communications, 470: 163–167.

Lv L., Yu K., Lü H., Zhang X., Sun C., Xu H., Zhang J., He X., Zhang D. (2020): Transcriptome-wide identification of novel

https://doi.org/10.17221/162/2022-HORTSCI

circular RNAs in soybean in response to low-phosphorus stress. PLoS One, 15: e0227243.

Nambeesan S., AbuQamar S., Laluk K., Mattoo A., Mickelbart M., Ferruzzi M., Mengiste T., Handa A. (2012): Polyamines attenuate ethylene-mediated defense responses to abrogate resistance to *Botrytis cinerea* in tomato. Plant Physiology, 158: 1034–1045.

Pirrung M. (1999): Ethylene biosynthesis from 1-Aminocyclopropanecarboxylic acid. Accounts of Chemical Research, 32: 711–718.

Song L., Fang Y., Chen L., Wang J., Chen X. (2021): Role of non-coding RNAs in plant immunity. Plant Communications, 2: 100180.

Sun JX., Lou Q., Peng YH., Yu L., Huang WT., Li LL. (2021): Effect of rhizosphere Ca^{2+} induced resistance to *Botrytis cinerea* in tomato. Journal of Shenyang Agricultural University, 52: 137–144.

Thrower J., Blalock R., Klinman J. (2011): Steady-state kinetics of substrate binding and iron release in tomato ACC oxidase. Biochemical Journal, 40: 9717–9724.

Wan P., Bao Y., Yee M., Barrent S., Hogan G., Olsen M., Dinneny J., Brown P., Salzman J. (2014): Circular RNA is expressed across the eukaryotic tree of life. PLoS One, 9: e90859.

Wang J., Yang Y., Jin L., Ling X., Liu T., Chen T., Ji T., Yu W., Zhang B. (2018): Re-analysis of long non-coding RNAs and prediction of circRNAs reveal their novel roles in susceptible tomato following TYLCV infection. BMC Plant Biology, 18: 104.

Wang P., Wang S., Wu Y., Nie W., Yiming A., Huang J., Ahmad I., Zhu B., Chen G. (2022): Identification and characterization of rice circular RNAs responding to *Xanthomonas oryzae* pv. *oryzae* invasion. Phytopathology, 112: 492–500.

Wang T., Wang G., Jia Z., Pan D., Zhang J., Guo Z. (2018): Transcriptome analysis of Kiwifruit in response to *Pseudomonas syringae* pv. *actinidiae* infection. International Journal of Molecular Sciences, 19: 373.

Wang W., Wang J., Wei Q., Li B., Zhong X., Hu T., Hu H., Bao C. (2019): Transcriptome-wide identification and characterization of circular RNAs in leaves of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) in response to calcium deficiency-induced tip-burn. Scientific Reports, 9: 14544.

Wang Y. (2019): Study on the role and molecular mechanism of tomato miR6024 in response to *Botrytis cinerea* infection. Master's Thesis, Zhejiang Sci-Tech University, Hangzhou, China.

Wang Y., Tyler B., Wang Y. (2019): Defense and counter defense during plant-pathogenic oomycete infection. Annual Review of Microbiology, 73: 667–696.

Yao S., Lu Z., Jin Z., Chen L. (2011): Analysis of spreading regularity and impact factors of *Botrytis cinerea* in greenhouse in the spring. Chinese Agricultural Science Bulletin, 27: 194–198.

Yuan M., Ngou B., Ding P., Xin X. (2021): PTI-ETI crosstalk: an integrative view of plant immunity. Current Opinion in Plant Biology, 62: 102030.

Zhou R., Xu L., Zhao L., Wang Y., Zhao T. (2018): Genome-wide identification of circRNAs involved in tomato fruit coloration. Biochemical And Biophysical Research Communications, 499: 466–469.

Zuo J. Wang Q., Zhu B., Luo Y., Gao L. (2016): Deciphering the roles of circRNAs on chilling injury in tomato. Biochemical and Biophysical Research Communications, 479: 132–138.

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