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RESEARCH

Transcriptome analysis of hot pepper plants identifies waterlogging resistance related genes

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ABSTRACT

Hot pepper (*Capsicum annuum* L.) is one of the most important vegetable crops in China, but floods bring substantial decreases in production over the past several decades. In order to investigate the mechanisms of waterlogging resistance in mutant hot pepper, we measured the agronomic traits of mutant and wild-type, as well as the activities of root antioxidant enzymes and the contents of osmotic regulation substance. At the same time, we did transcriptome sequencing on the plant roots, and screened for differentially expressed genes between mutant and wild-type. The results showed that, under waterlogging stress, the mutants could grow normally, and the activities of their superoxide dismutase, peroxidase, catalase, and glutathione reductase were significantly increased, as well as the contents of proline and soluble sugar. The accumulation of malondialdehyde and hydroxyl radical in mutants was significantly reduced. Among the 61 differentially expressed genes from transcriptome analysis, 24 genes were up-regulated and 37 genes were down-regulated in mutants. After functional analysis, we found 8 genes related to the metabolism of endogenous hormone and protective enzymes, among which, auxin-induced protein related gene *cap.ARATH*, ethylene response related gene *Cap.RAP2*, MYB family related gene *Cap.MYB1R1*, and the 4 genes related to peroxidase *Cap.POD*, were significantly up-regulated in mutants, while the *Capana01g001329* gene was down-regulated. These results suggest that under waterlogging stress, the mutant could enhance its resistance to waterlogging by regulating the genes involved in metabolism of endogenous hormones and protective enzymes. This study provides the scientific basis for elucidating the gene regulation network of mutant pepper under waterlogging stress.

Key words: *Capsicum annuum*, gene, hot pepper, mutant, transcriptomic data, waterlogging.

INTRODUCTION

Pepper (*Capsicum annuum* L.) belongs to *Solanaceae* family, *Capsicum* genus. It is an annual herb or limited perennial herb. Pepper originates from the tropical areas in middle Latin America, and is widely used in vegetables and spices, as well as in other applications such as medicines, natural colors, cosmetics and ornamental plants. Also, pepper is one of the most important vegetable species in China, with the annual planting area of more than 1.3 million ha (Zou, 2009). Pepper has strong drought tolerance (Ou et al., 2012), but its ability to resist waterlogging is weak (Ou et al., 2011). It is easy to catch virus disease during rainy seasons, resulting in serious flower and fruit falling. Sometimes, too many fallen leaves even cause the plant death. In China, 60%-70% of the peppers are growing in open field. In many pepper production areas, the agricultural infrastructure such as irrigation is incomplete, so the peppers are often subjected to waterlogging and other disasters, bringing great losses to pepper production and quality. Moreover, it is very difficult to find waterlogging-resistant peppers in routine breeding, so the discovery or accumulation of waterlogging resistant genes is nearly impossible.

The important ways to improve crop waterlogging resistance are discovering new waterlogging resistant genes and introducing them into crop genome and using molecular markers to select and accumulate resistant genes (Valliyodan et al., 2017). Zou et al. (2013) examined the gene expression profiles of the roots from waterlogging resistant rape seedlings after 12 h of waterlogging treatment, and found 4432 differentially expressed genes. They analyzed the 200 highly expressed genes, and found 144 of them were down-regulated under waterlogging stress. Moreover, they identified 7 genes encoding for ethylene responsive factors (ERF) were regulated upon waterlogging stress, and 5 of them, including ERF2, ERF4, ERF7, ERF11 and ERF54, were induced by stress. Lee et al. (2014) analyzed the gene expression profiles of rapeseed leaves after 36 and 72 h of waterlogging stress, and found that compared to control, the photosynthesis related genes were down-regulated, and the genes related to scavenging reactive oxygen, protein, starch and lipid degradation were up-regulated.

An effective way to improve plant qualities is mutagenesis treatment via physical or chemical factors, which has been widely used in crops, gardening, flowers, etc. Ethyl methanesulfonate (EMS) mutagenesis is one of the commonly used methods to obtain mutants manually, and is more specific compared to other mutagenesis methods. The EMS target is DNA base, causing base changes such as substitution and reversion. Since the mutants generated by EMS usually only have alterations on individual bases, they have simple backgrounds as compared to the extreme cases obtained from conventional screening, thereby they can reveal the related regulatory networks in a better way. There have been studies using the mutants obtained from EMS mutagenesis to investigate the salt tolerance (Pei et al., 2017), resistance to disease (Wu et al., 2008; Xu et al., 2017a), low temperature tolerance (Jin et al., 2003) and drought tolerance (Zhang et al., 2011) in rice, alfalfa, Chinese wolfberry, bilberry and other species. So far, due to the lack of mutants, the selection of waterlogging resistant pepper and gene analysis on related functions mainly depend on conventional methods, primarily focusing on phenotypical, physiological and biochemical parameters. In 2013, our group mutated 'S15' via EMS, and found a mutant with strong waterlogging resistance from screening, named 'RW15'. In this paper, we treated the mutant and wild-type with waterlogging stress, and compared their differences in basic agronomic traits, antioxidant enzyme activities, and the content of osmotic regulation substance. We also sequenced the transcriptomes of their roots, and analyzed the differences in gene expression and regulation, in order to find the important genes involved in waterlogging stress response and elucidate the mechanisms of waterlogging resistance in mutants.

MATERIALS AND METHODS

Plant materials and stress conditions

In 2013, we obtained the M_1 generation from EMS processed 'S15', and then acquired the M_2 generation after self-crossing. After conducting waterlogging screening on M_2 generation, we obtained a waterlogging resistant mutant strain, and self-crossed it to get M_3 seeds. The seeds of M_3 and wild-type (WT) peppers were germinated and planted in seedling tray. At the 5-leaf-1-heart phase, the robust seedlings with consistent growth rate were planted in 9 cm × 9 cm nursery pots, 1 seedling per pot. The pots were filled with soil until 1 cm to the edge, and the soil contained 60% farmland soil, 20% cow dung compost, and 20% river mud. When seedlings grew to the 6-leaf-1-heart period, they were subjected to waterlogging stress using dual-pot method. The nursery pots were put into a 15 cm × 40 cm larger pot, each larger pot included 6 pots (3 mutants, 3 WT). The water level at 2 cm above nursery pot surface was considered waterlogging. The water was supplemented every other day to maintain the flooding level, and the waterlogging treatment was repeated for three times. Ten days after the treatment, leaves and roots were washed with clean water, wiped, and quickly frozen with liquid nitrogen. The samples were stored in -80 °C freezer for future use.

Agronomic characterization

The height and root length of randomly selected pepper plants were measured with a ruler. The dry weight and fresh weight of over-ground part, underground part and the entire plant were recorded. Then the plants were killed at 105 °C for 30 min, and dried at 75 °C until constant weight.

Measurements of enzyme activity and plant inclusions

Proline content was measured by the acid ninhydrin method. The fresh leaves were washed and dried with paper towel.

Samples of leaves (0.5 g) were cut into pieces, mixed with 5 mL 3% sulfosalicylic acid solution in a large tube, and boiled for 10 min under shaking. The extract was filtered with a funnel into a clean tube after cooling down, 2 mL filtered extract was mixed with 2 mL acetic acid and 2 mL acid ninhydrin, and boiled for 30 min after sealed with plastic wrap. After cooling down, 4 mL toluene was added into the tube and fully oscillated. The upper red solution was collected and its absorption at 520 nm was measured using toluene as reference. Proline content [x (μg mL^{-1})] in the 2 mL tested samples was obtained from the standard curve and the proline concentration in fresh leaves was calculated based on the following formula: Proline content (μg g^{-1}) = (x × 5/2)/fresh leaf weight (g).

Anthrone method was used to measure soluble sugar content. They were measured with anthrone colorimetry. Samples of leaves (0.1 g) were placed in a ground glass weighing bottle, mixed with 20 mL distilled water, and extracted twice in boiling water for 30 min. The extract was filtered into a 50 mL volumetric flask, and the weighing bottle and residues were washed repeatedly before being filled to constant volume. Sample extract (0.5 mL) was mixed thoroughly with 1.5 mL distilled water, 0.5 mL anthrone ethyl acetate solution and 5 mL concentrated sulfuric acid, before being immediately placed in boiling water bath for 1 min. The heated sample extract was cooled naturally to room temperature to determine the absorbance at 630 nm. The final soluble sugar contents were calculated from the standard curve: soluble sugar (μg g^{-1} FW) = [(corresponding sucrose content from standard curve (μg) × total extract volume (mL)]/[(measurement volume (mL) × fresh weight of the sample (g)].

The content of malondialdehyde (MDA) was determined by thiobarbituric acid (TBA method) by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing China). Samples of leaves (0.1 g) were thoroughly ground with a cold mortar and pestle in an ice bath. The grinding medium was 4 mL saline, plus homogenizing glass beads. The homogenate was centrifuged for 10 min at 2500 rpm and 25 °C. The supernatant referred was crude enzyme extract and used to determination. The absorbance of the reaction mixture was determined by using a spectrophotometer (UV-1780, Shimadzu, Kyoto, Japan).

The colorimetric assay was used to measure the contents of peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GS) and hydroxyl free radicals (·OH) (the kit was purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing Shi, China) (Yang et al., 2016). A sample of leaves (0.1 g) without midrib were thoroughly ground with a cold mortar and pestle in an ice bath. The grinding medium was 4 mL saline, plus homogenizing glass beads. The homogenate was centrifuged for 10 min at 2500 rpm and 4 °C. The supernatant referred was crude enzyme extract and used to determination. The absorbance of the reaction mixture was determined by using a UV-1780 spectrophotometry (Shimadzu, Japan).

RNA Extraction, library preparation and sequencing

Total RNA was extracted from tissues using TRI Reagent (Sigma Life Science, St. Louis, Missouri, USA), according to manufacturer's instructions. RNA quality was checked by RNase-free agarose gel electrophoresis to avoid possible degradation and contamination, and then verified using Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, California, USA). Next, Poly (A) mRNA was isolated using oligo-dT beads (Qiagen, Hilden, Germany), and then broken into short fragments by adding fragmentation buffer. First-strand cDNA was synthesized using random hexamer-primed reverse transcription, followed by the synthesis of the second-strand cDNA using RNase H and DNA polymerase I. The cDNA fragments were purified using a QIA quick PCR extraction kit, and then washed with EB buffer for end reparation poly (A) addition and ligated to sequencing adapters. Following agarose gel electrophoresis and extraction of cDNA from gels, the cDNA fragments were purified and enriched by PCR to construct the final cDNA library, which was then sequenced on the sequencing platform (HiSeq X Ten, Illumina, San Diego, California, USA) using the paired-end technology. Three biological replicates were performed for each line, thus six libraries were generated and sequenced.

Transcriptome analysis

Raw reads were filtered to remove low quality sequences (there were more than 50% bases with quality lower than 20 in one sequence), reads with more than 5% N bases (bases unknown) and reads containing adaptor sequences through the Perl program. Then the clean reads were mapped to the tomato reference genome using TopHat2 (http://ccb.jhu.edu/software/tophat), with the following parameters: -min-intron-length 20 -max-intron-length 10 000 -read-mismatches 1. The differentially expressed genes (DEGs) were identified using the R package edgeR (R Foundation for Statistical

Computing, Vienna, Austria). The expression level of each gene was calculated and normalized to FPKM (fragments per kilobase of transcript per million fragments mapped). The false discovery rate (FDR) was used to determine the threshold of the P-value in multiple tests. In our study, the FDR < 0.05 and fold change > 2 were used as significance cut-offs of the gene expression differences. The DEGs were used for Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analyses according to Tan et al. (2018); GO terms with corrected P-value < 0.05 and KEGG pathways with P-value < 0.05 were considered significantly enriched by differential expressed genes.

Quantitative real-time PCR

The total root RNA was extracted using TaKaRa MiniBEST Plant RNA Extraction Kit (Takara Bio, Kusatsu, Shiga, Japan), followed by reverse transcription to obtain a total of 200 ng cDNA. qRT-PCR based on SYBR method was used to validate the DEGs (FastStart Essential DNA Green Master, Roche, Basel, Switzerland), *CpAction* was used as the internal reference gene (Liu et al., 2017). The qPCR primers (Table 1) were synthesized by Shanghai Biotech (Shanghai, China). PCR reaction system was 20 µL: Master mix 10 µL, forward and reverse primers 0.5 µL each, 10 times diluted cDNA 5.0 µL, H_2O 4 µL. The qPCR cycle was: 95 °C for 10 min, 95 °C 15 s, 60 °C 30 s, 72 °C 15 s for 45 cycles.

Data analysis

SPSS17.0 (IBM, Armonk, New York, USA) was used to conduct one-way ANOVA. LSD and Duncan's method were used for pairwise comparisons. P < 0.05 was considered significant.

RESULTS

Phenotypic evaluation of experimental materials

Under waterlogging stress, root length, plant height, fresh and dry weights of entire plant were all affected. Moreover, there were significant differences between mutant and WT after waterlogging treatment. The root length, plant height, fresh weight and dry weight of the mutants were 78.30%, 17.68%, 83.84% and 105.19% higher than WT, respectively (Table 2).

Table 1. Information of primer sequences.

Gene ID-accession	Primer sequence
Capana04g001523	F:TTCTTGTTACCTCTGCTCAA
	R:CGCTGGATGAATTGTAGTTC
Capana08g000001	F:TTCAGCAAGTCGTCATCAA
	R:TTCACTCCATTCACAGCATA
Capana01g001329	F:TGCCAGCCACAATCTTAG
	R:GTCTACAATAGCGGAAGGAT
Capana12g002272	F:TTGAGGTGATTGATGCTGTA
	R:GAATGGAGAAGGAAGATTGTC
Capana02g003649	F:TCAACAAGGAGGTTCTACTG
	R:GACAGCACAACATTATCCAA
Capana03g002143	F:TCACGACTCTTACCTTGTAG
	R:TCCAACTTCCAACGCATT
Capana12g002274	F:TTGAGGTGATTGATGCTGTA
	R:GAATGGAGAAGGAAGATTGTC
Capana05g002019	F:ATGGACAGAGGAGGAACA
	R:AGATTGACGGTGGTGATG
CpAction	F:CCACCTCTTCACTCTCTGCTCT
	R:ACTAGGAAAAACAGCCCTTGGT

Table 2. Agronomic traits of pepper under waterlogging stress.

	Root length	Plant height	Fresh weight	Dry weight
	cm		g	
Wild-type	4.47 ± 0.15b	12.67 ± 0.28b	1.12 ± 0.03b	0.15 ± 0.0011b
Mutant	7.97 ± 0.32a	14.91 ± 0.33a	2.07 ± 0.05a	0.31 ± 0.0026a

Each value is the mean ± standard deviation (SD, n = 6). The effects are significant at p < 0.05 with a one-way ANOVA.

Physiological response of pepper to waterlogging stress

In addition, under waterlogging stress, the antioxidant enzymes in mutants showed higher activities: SOD, POD, CAT and GR enzymes of mutants were 90.61%, 77.92%, 43.05% and 106.67% more active than WT, respectively (Table 3). Meanwhile, the contents of proline and soluble sugar in mutants were 25.53% and 42.08% higher than WT (Table 4). And the contents of MDA and ·OH were reduced in mutants by 47.54% and 68.72% compared to WT (Table 4).

RNA-seq analysis

Transcriptome sequencing was performed by Hiseq X ten. After eliminating the joint and low-quality sequences, we obtained 211991416 clean and high-quality sequences with a total of 31.79 Gb data. Among these sequences, 1666942 723 of them were uniquely matched to the pepper genome. The percentage of Q30 bases were 89.67%-90.64%, and the average GC content was about 45%, indicating that the quality of transcriptome sequencing was relatively high, with good reproducibility between different replicates, which satisfied the criteria for further bioinformatic analysis (Table 5).

GO functional annotation enrichment analysis

Under waterlogging stress, 61 DEGs were detected between mutant and wild-type. Among them, 37 DEGs were down-regulated in mutant, and 24 DEGs were up-regulated (Figure 1). After conducting the GO functional annotation analysis on differentially expressed genes, we found that these genes were concentrated in three major categories, biological processes, molecular functions and cell components (Figure 2), which included 6, 5 and 5 functional classes, respectively. The percentages of differential genes in these three categories were 45%, 35%, and 20%, respectively, Mutant and wild type had the largest differentially genes in single organism processes, antioxidant activity and membrane of this three categories, respectively, which indicated that most DEGs were significantly related to some of these biological functions. Indicating that most of these genes were significantly associated with some biological functions.

Table 3. Activities of antioxidant enzymes of pepper under waterlogging.

	SOD	POD	CAT	GR
		U mg^{-1} prot		U g^{-1} prot
Wild-type	64.85 ± 5.36b	96.69 ± 10.25b	38.91 ± 3.62b	0.15 ± 0.01b
Mutant	123.61 ± 10.39a	172.03 ± 18.74a	55.66 ± 4.89a	0.31 ± 0.02a

Each value is the mean ± standard deviation (SD, n = 6). The effects are significant at $p < 0.05$ with a one-way ANOVA.
SOD: Superoxide dismutase; POD: peroxidase; CAT: catalase; GR: glutathione reductase.

Table 4. Contents of osmotic substances and oxidation products of pepper under waterlogging.

	PRO	Soluble sugar	MDA	·OH
	μg g^{-1} FW	mg g^{-1} FW	nmol mg^{-1} prot	U mg^{-1} prot
Wild-type	45.95 ± 3.98b	23.17 ± 1.92b	71.24 ± 6.65a	30.75 ± 3.25a
Mutant	57.68 ± 4.85a	32.92 ± 2.84a	37.37 ± 3.25b	9.62 ± 0.87b

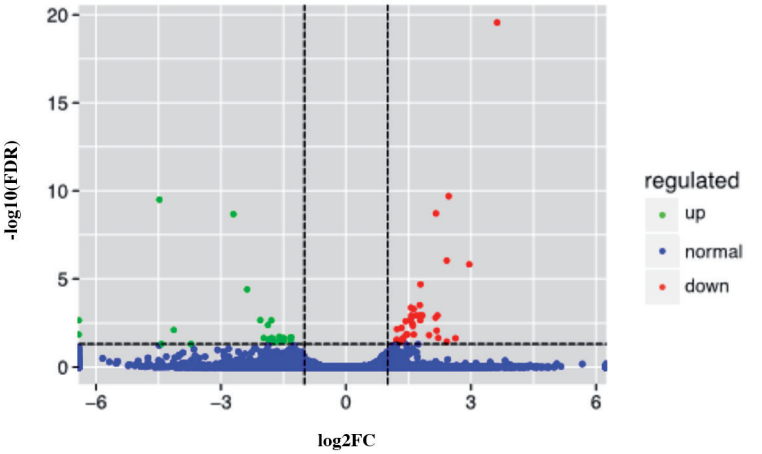
Each value is the mean ± standard deviation (SD, n = 6). The effects are significant at $p < 0.05$ with a one-way ANOVA.
PRO: Proline; MDA: malondialdehyde; ·OH: hydroxyl free radical.

Table 5. RNAseq data statistics.

Group	Sample ID	Number of input reads	Input base (Gb)	Uniquely mapped reads	GC content %	Bases Q20%	Bases Q30%
Wild-type	7R4351Q2B361PB	35752891	5.36	28219648	45.92	95.85	90.60
	7R4352Q2B362PB	33306020	5.00	27558517	45.43	95.36	89.68
	7R4355Q2B363PB	36496662	5.47	28154324	46.41	95.26	89.44
Mutant	7R4344Q2B358PA	35821154	5.37	30787325	44.52	95.72	90.24
	7R4347Q2B359PA	38258824	5.74	25517669	47.45	95.91	90.64
	7R4349Q2B360PA	32355865	4.85	26705240	44.95	95.37	89.67
Total		211991416	31.79	166942723			

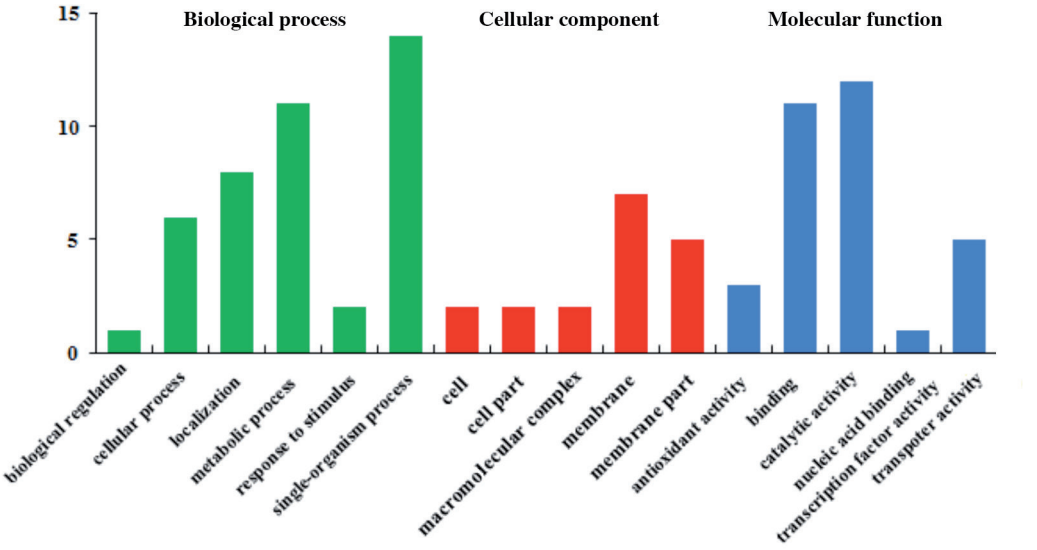
GC: Ratio of the sum of guanine and cytosine; Q20: Ratio of bases with a mass value greater than or equal to 20; Q30: Ratio of bases with a mass value greater than or equal to 30.

Figure 1. Differential expression genes.



The fold changes (Mutant (Mut)/Wild-type (WT), in logarithm scale) were represented by y-axis (-log10 of the qvalue). Each dot in the figure represents a single gene, and the green and red dots represent the significantly differential genes. Green dot indicates an up-regulated gene (Mut/WT), and red dot indicates a down-regulated gene (Mut/WT). Blue dots represent genes with nonsignificant difference.

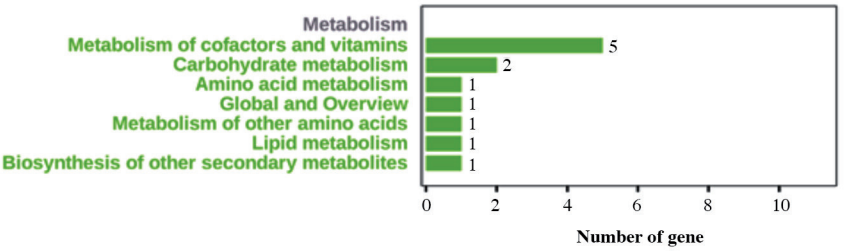
Figure 2. Gene ontology annotation of differentially expressed genes.



KEGG Pathway analysis of DEGs

The KEGG enrichment analysis on DEGs showed that, 12 out of the 61 genes were annotated to seven pathways, including the cofactors and vitamin metabolism, amino acid metabolism, lipid metabolism, etc. (Figure 3).

Figure 3. KEGG annotation of differentially expressed genes.



KEGG: Kyoto Encyclopedia of Genes and Genomes.

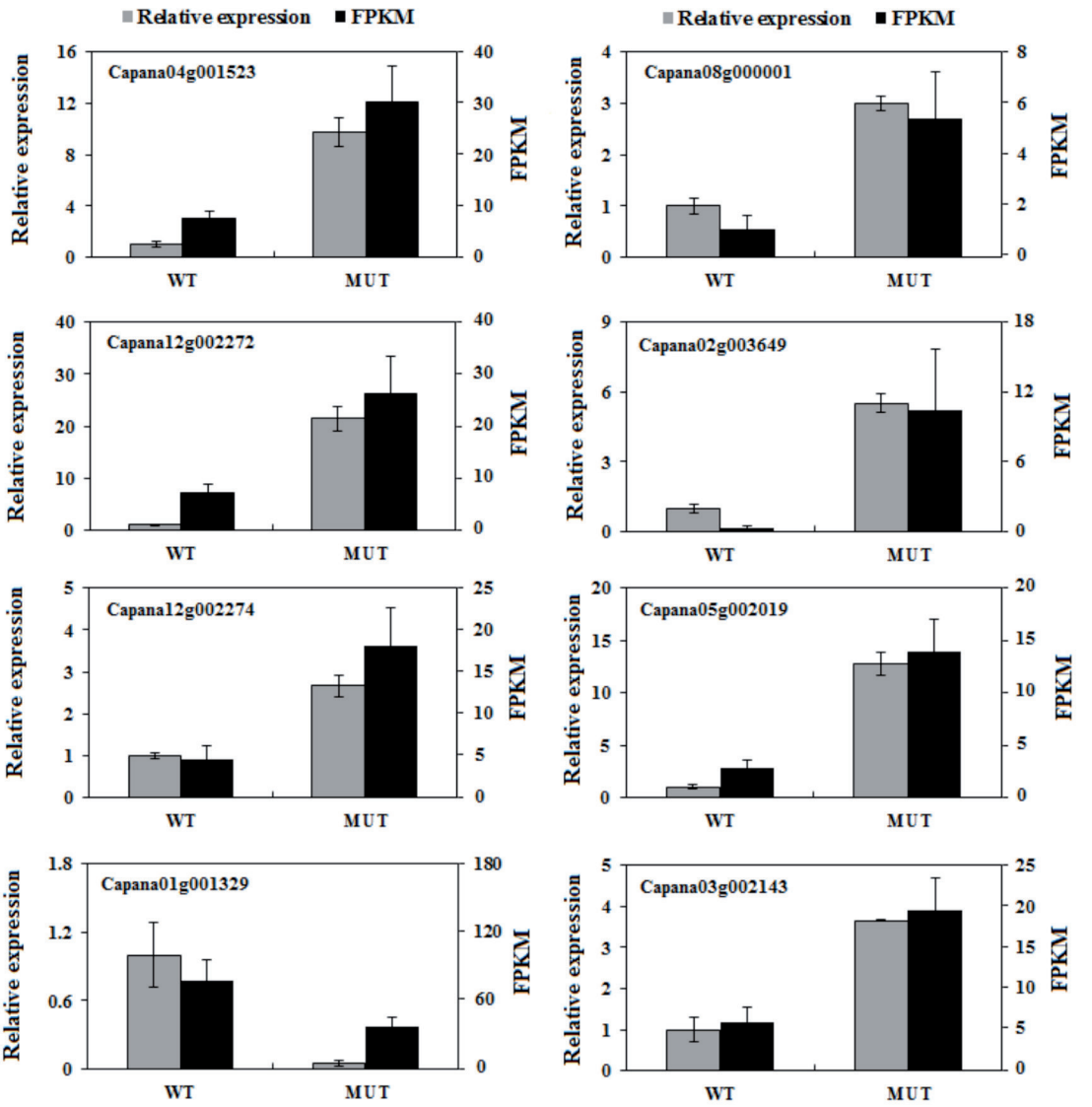
Screening of DEGs

Among the DEGs selected from transcriptome sequencing data, we identified 8 genes related to the protective enzymes and endogenous hormone metabolism, including one auxin-induced protein related gene *Cap.ARATH* (Capana04g001523), one ethylene response related gene *Cap.RAP2* (Capana08g000001), five peroxidase related genes *Cap.POD* (Capana02g003649, Capana12g002272, Capana01g001329, Capana03g002143, and Capana12g002274), and one MYB family related gene *Cap.MYB1R1* (Capana05g002019).

Quantitative verification of the critical genes

The expression levels of eight genes related to protective enzymes and endogenous hormone metabolism were quantified, and found to be significantly different between mutant and WT (Figure 4). Among them, the expression levels of auxin-induced protein related gene *Cap.ARATH* (Capana04g001523) and ethylene response related gene *Cap.RAP2* (Capana08g000001) were significantly higher in mutant. The peroxidase related genes *Cap.POD* (Capana12g002272, Capana02g003649, Capana03g002143 and Capana12g002274) were up-regulated in mutants to different extents,

Figure 4. Relative gene expression and FPKM level.



FPKM: Fragments per kilobase of transcript per million fragments mapped; WT: wild-type; Mut: mutant.

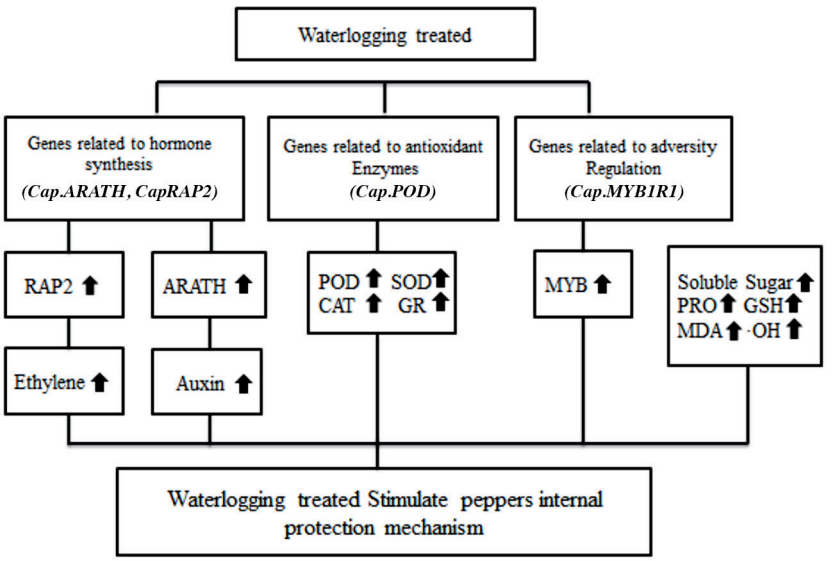
which was consistent with the changes in POD activity; but another peroxidase related gene (Capana01g001329) was significantly down-regulated in mutants compared to WT. The expression level of MYB family related gene *Cap.MYB1R1* (Capana05g002019) was also significantly increased in mutant.

DISCUSSION

Numerous studies have shown that waterlogging stress can cause physiological and biochemical changes in plant, leading to inhibited growth and development (Ullanat and Jayabaskaran, 2002; Perata and Voesenek, 2007; Vidoz et al., 2010; Qi et al., 2011). Under waterlogging stress, the root environment easily becomes hypoxic, which hinders the oxygen supply, resulting in suppression of mitochondrial ATP synthesis and NADH oxidation. Thereby the plant cannot maintain energy metabolism through normal aerobic respiration and switch to anaerobic respiration, which costs more organic material, generates less energy, and produces harmful metabolites such as ethanol, leading to metabolic disorders and growth inhibition in plant. MDA and ·OH are the lower oxidation products of the free radical chain reaction caused by the continuous accumulation of ROS. Its content reflects the toxic level of ROS to plants, which can deactivate the proteins and biological enzymes on the cell membrane, destroy the structure and function of the biofilm, and affect the normal material metabolism of cells. In this study, we found that the waterlogging resistant mutant exhibited significantly higher activities of antioxidant enzymes and more osmotic regulation substance under waterlogging treatment, as well as less MDA and ·OH. These results suggest that the waterlogging stress can cause reduced antioxidant enzyme activity and osmotic regulation substance of pepper root system, and the mutants are able to maintain high enzyme activity and osmotic substance content under this condition, in order to resist the waterlogging environment.

The physiological and biochemical changes of plant under waterlogging stress are actually a series of chain changes caused by the altered gene expression in the related metabolic pathways (Figure 5). As a result of these changes, the content of antioxidant enzymes, proline, soluble sugar, glutathione and other metabolites increased, thus enhancing the waterlogging resistance of plants. Zou et al. (2010) used suppression subtractive hybridization (SSH) and found that many genes were upregulated in maize under flooding conditions, and 66 genes were located close to the known quantitative trait locus (QTL). Du et al. (2014; 2016) used reverse genetics and bioinformatics to explore the AP2/ERF and GT transcription factors associated with maize waterlogging resistance. There is also a study showing that *Sub1A* gene is a key regulator for waterlogging resistance, and it can effectively reflect ethylene production and response

Figure 5. The mechanism diagram of waterlogging stress in mutant.



SOD: Superoxide dismutase; POD: peroxidase; CAT: catalase; GR: glutathione reductase; PRO: proline; GSH: glutathione; MDA: malondialdehyde; ·OH: hydroxyl radical.

(Damanik et al., 2010) through bud elongation and energy consumption (including carbohydrates, fats and proteins) during and after flooding; overexpressing of *Sub1A* in rice can enhance the waterlogging resistance (Xu et al., 2006; Fukao and Bailey-Serres, 2008). Low oxygen condition promotes the ethylene synthesis in root system (Atwell et al., 1988). For example, under waterlogging stress, tomato can induce adventitious root formation by regulating the expression of *ACS*, a key gene for ethylene synthesis, through ethylene-responsive elements (ERFs), and promoting the interaction between ethylene and auxin (Vidoz et al., 2010). Hattori et al. (2009) reported that under waterlogging stress, two genes *SK1* and *SK2* interacted with ethylene-responsive elements (ERFs) in the ethylene signal transduction pathway, thereby triggering the GA involved stem elongation and growth. In this study, transcriptome analysis revealed that the auxin-induced protein related gene *Cap.ARATH* and ethylene response related gene *Cap.RAP2*, which can promote auxin and ethylene content, were significantly upregulated in mutant, indicating that the mutations in mutants may be helpful to increase the auxin and ethylene synthesis of plant root system under waterlogging stress, and maintain the normal growth of pepper by increasing the contents of auxin and ethylene.

Studies have shown that MYB transcription factors are related to the plant morphogenesis (Zhang et al., 2009; Brownfield et al., 2009) and metabolic regulation of many primary products and secondary products (Cone et al., 1986; Song et al., 2011; Peng and Moriguchi, 2013; Tombuloglu et al., 2013) such as anthocyanins and phenylpropanoids, which are closely related to plant resistance. In this study, we found that the expression of *Cap.MYB1R1* was significantly increased in mutants under waterlogging stress. *Cap.MYB1R1* is an important transcription factor gene, and its increased expression helps to control the production of related metabolites to enhance the plant waterlogging resistance. Waterlogging can cause the formation of large numbers of aerenchyma in root system, leading to the decrease in plant lignin content (Tao et al., 1998; Chen et al., 2015). Also, certain genes in plant can participate in multiple metabolic regulation pathways (Arce-Rodríguez and Ochoa-Alejo, 2017). Our study found that *Capana01g001329* not only participates in the regulation of POD enzyme, but also takes part in the regulation of lignin biosynthesis. The expression of *Capana01g001329* in mutants was significantly decreased, which may due to its involvement in regulating lignin synthesis. The reduced root lignin causes regular separation and differentiation of root cells or programmed cell death to form hollow cavities, which, to some extent, promotes the aerenchyma formation in plant root and enhances the waterlogging resistance.

Xu et al. (2017b) found that in cucumber, the GO terms 'response to chemical stimulus', 'oxidoreductase activity', 'regulation of cellular process' and 'regulation of metabolic process' were highly enriched after waterlogging stress. Qi et al. (2012) and Christianson et al. (2010) analyzed the differentially expressed genes of cucumber and cotton roots after waterlogging, and found that the differential genes in cucumber were mainly involved in C cycle, photosynthesis, generation and elimination of ROS, and hormone-mediated signaling pathways, while the differential genes of cotton were mainly involved in cell wall growth, fermentation pathway, hormone signal response and C-N metabolism, In this study, the transcriptome sequencing also identified several genes associated with single-organism processes, metabolic processes, and catalytic processes, etc.; indicating that different plants had different waterlogging resistant mechanisms. Therefore, the DEGs we identified by comparing pepper mutants and wild-type after waterlogging stress provide important information for understanding the waterlogging damage and resistance mechanism of pepper.

CONCLUSIONS

In conclusion, we proposed a model of the protective mechanism of mutant pepper against waterlogging stress. Waterlogging stress can induce the synthesis of related hormones and altered expression of antioxidant enzymes in root system, leading to the changes in some endogenous hormones and protective enzymes, which cause the growth inhibition and even plant death. However, the waterlogging resistance mutants can effectively up-regulate the expression of *Cap.ARATH*, *Cap.RAP2*, *Cap.MYB1R1*, and *Cap.POD* expression thus increasing the activities of endogenous hormones (ethylene and auxin), antioxidant enzymes (peroxidase, superoxide dismutase, catalase, glutathione reductase) and content of related inclusions (soluble sugar, proline and glutathione) under waterlogging stress and maintaining the plant growth and development.

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