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Special Research Report #310: Development of Petunia with Enhanced Drought Stress Tolerance

Plant Breeding and Genetic Engineering

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BACKGROUND

Ornamental crops often experience abiotic stresses (heat and water deficit stress) during shipping and/or in the retail environment. Water deficit stress is one of the most devastating stresses to agricultural crops. Total crop damage from drought in 2016 was in the billions of dollars (NOAA, 2017) and up to 20% of the ornamental crops are estimated to be unsaleable due to the poor postproduction environment which includes water deficit conditions (Armitage, 1993).

Understandably, intensive research has been focused on developing crops that are tolerant to water deficit stress. Until recently, only Monsanto offered a hybrid corn (Genuity® DroughtGard Hybrids) with a world's first and only drought tolerant biotechnology trait (Monsanto, 2017). However, DroughtGard corn was developed using a bacterial *cspB* gene combined with the conventional breeding program (DiLeo, 2015). Currently no crop with drought tolerance has been genetically engineered using a gene cloned from plant sources is on the market.

Multiple genes are known to regulate water deficit tolerance that help plants to withstand the adverse conditions. Some of the genes, encoding transcription factors, (TF) are involved in abscisic acid (ABA) dependent or independent pathway. When plants perceive water deficit, ABA is synthesized and acts as a signaling messenger to activate a certain group of regulatory genes such as kinases and TFs. TFs in turn activate or inactivate downstream genes that are involved in water deficit stress pathways. Therefore, manipulation of TFs that respond to the water deficit stress at the early stage of the stress could enhance the tolerance.

MATERIALS & METHODS

1. RNA sequencing

Eighteen petunias were grown in 10-cm pots containing soilless media (Sunshine Mix #1: Sun Gro Horticulture, Agawam, MA) and a half of them were irrigated daily (control), while the other half of the petunias (stressed) received no water. Petunia leaves from both treatments were collected 1, 3 and 5 days after treatments. Total RNAs were isolated and complementary DNA (cDNA) libraries were constructed for each sample. All cDNAs were aligned to identify unique genes (contigs). The names of genes were identified based upon sequence similarity of other known genes.

Differentially expressed genes by two folds, either up- or down-regulated, were selected for further analyses.

2. Transcriptome analysis

Genes encoding TFs, kinases and phosphatases that are known to regulate biological processes were selected. To decrease the number of genes to a manageable size for functional analyses, genes that were already known to be involved in water deficit stress response mechanism, and expressed later stage of stress (day 5) were eliminated. For further reduction of the number of genes, only upregulated genes encoding TFs that were speculated to be involved in hormone or stress response mechanism were selected.

3. Functional analysis

Selected candidate genes were cloned for over-/under expression analyses using vectors purchased from Addgene. Validation of the gene insertion/deletion was conducted by gel analyses. Expression level of the gene of interest is currently being measured using semi-quantitative RT-PCR. Water deficit tolerance of the transgenic petunias will be evaluated using an automated irrigation system (Nemali and van Iersel, 2006). Media moisture contents are set at 10, 20, 30 and 40%. Visual wilting symptom and stomatal conductance of the transgenic petunias will be measured and compared to those of the control petunias. The evaluation will be repeated with 2nd generation of transgenic petunias.

RESULTS

1. RNA sequencing and transcriptome analysis

Our study revealed that the levels of mRNA of more than 6,000 genes were differentially expressed during water deficit stress (Figure 1). Among them, 3,604 genes produced more mRNA (upregulated), while 2,580 genes synthesized less than normal level of mRNA (downregulated). Petunias responded to the water deficit stress the most actively 3 days after treatment based upon the gene expression pattern.

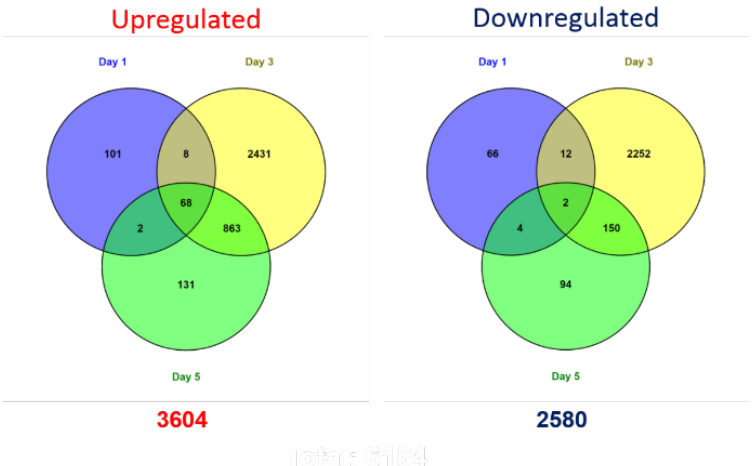

Figure 1. Total number of genes that were differentially expressed during 5 days of water deficit stress in petunia. The total number of differentially expressed genes was 6,184.

Of 6,184 differentially expressed genes, some genes are known to be involved in regulation of the biological processes. These genes encode TFs, kinases and phosphatases. In our study 366 regulatory genes were identified (Table 1) and there were 94 FTs, 230 kinases and 42 phosphatases. The largest number of the regulatory genes were detected 3 days after treatment.

Table 1. Number of genes that were either upregulated or downregulated among regulatory genes during water deficit stress treatment in petunia.

Expression	Regulatory Genes	Day 1	Day 3	Day 5	Unique Genes[z]
Upregulated	Transcription Factors	2	40	8	46
	Kinases	3	117	18	126
	Phosphatases	5	0	6	11
Downregulated	Transcription Factors	1	44	5	48
	Kinases	3	96	7	104
	Phosphatases	0	30	2	31
Total		14	327	46	366

[z]*Number of unique genes excluded the duplicated genes found in different days of treatment.*

Of 366 potential candidate regulatory genes, six genes were selected. The selection criteria were transcription factors that were upregulated at the early stage of stress response (1 and/or 3 days after treatment). The number of TFs was further reduced by eliminating TFs that had been already studied in water stress tolerance. Genes encoding TFs that were downregulated, and kinases and phosphatases will be evaluated in the later experiment. The final candidate genes are shown in Table 2.

Table 2. List of candidate transcription factors.

Candidate gene	Length (bp)	Upregulated date	$\log_2$ (fold change)
ethylene insensitive 3-like 3 protein (EI3P)	2025	Day 3	3.43
abscisic acid-insensitive 5-like protein 7 (ABI5)	1233	Day 3 and 5	2.13/1.66

heat stress transcription factor b-2b (HSFB2B)	915	Day 3 and 5	1.95/1.86
ethylene-responsive transcription factor erf039 (ERF039)	816	Day 3	1.60
ap2-like ethylene-responsive transcription factor (ERF)	1278	Day 3	1.32
dehydration-responsive element-binding protein 3 (DREB3)	573	Day 3	3.41

2. Functional analysis

Of six TFs, transgenic petunias harboring an extra copy of *PhERF039* were obtained (Figures 2 & 3). *PhERF039* was successfully inserted into petunia genome (Figure 2). Six positive transgenic petunias have been generated and initially validated by the expected size of *PhERF039* (816 bp). Five of these plants have been transplanted into growing media (Figure 3). Generation of transgenic petunias with loss of function (*PhERF039*) is currently underway using CRISPR/Cas9 technology. The rest of 5 candidate genes are cloned.

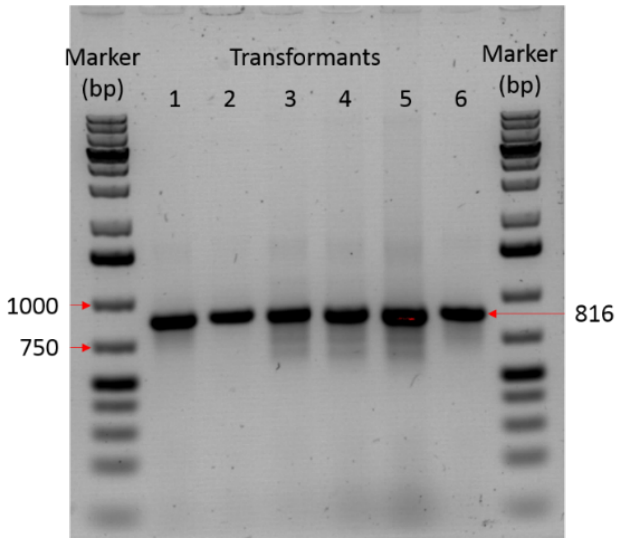

Figure 2. Verification of the insertion of PhERF039

Figure 3. Transgenic petunias containing an extra copy of PhERF039

CONCLUSIONS

RNA sequencing was conducted to identify genes that play a role in regulating water deficit stress tolerance. More than 6,000 genes were differentially expressed during water deficit stress. Among them, six candidate genes encoding transcription factors were selected through transcriptome analyses. In addition, transgenic petunias harboring one of candidate genes (*PhERF039*) were generated to evaluate the function of the gene. In parallel, five other candidate genes are being cloned for the functional analyses. Cloning and functional analyses will continue until a transgenic petunia which shows a significant tolerance to water deficit stress.

INDUSTRY IMPACT

Development of genetically modified crops with enhanced tolerance to water deficit stress is one of the most challenging research projects, and yet it is the most sustainable approach with a significant economic benefit. Crops with water deficit tolerance will withstand unfavorable conditions during shipping and retailing. In addition, genetically engineered crops with enhanced water deficit tolerance traits could exhibit salt or cold tolerance because similar mechanisms were discovered in salt or cold stress response. The knowledge obtained from this research could be easily applied for other crops in the same family such as tomatoes, potatoes and eggplants.

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