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Original Research Article

Identification of Candidate Transcripts Related to Drought Stress using Secondary Traits and qRT-PCR in Tropical Maize (*Zea mays* L.)

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ABSTRACT Global climate change exerts adverse effects on maize production. Among abiotic stresses, drought stress during the tasseling stage (VT) can increase anthesis-silking intervals (ASI) and decrease yield. We performed an evaluation of ASI and yield using a drought-sensitive line (Ki3) and a drought-tolerant line (Ki11) to analyze the correlation with ASI and yield. Moreover, the *de novo* data of Ki11 were analyzed to find putative novel transcripts related todrought stress in tropical maize. A total of 182 transcripts, with a log2 ratio >1.5, were found by comparing drought conditions to a control. The top 40 transcripts of high expression levels in the *de novo* analysis were selected and analyzed with PCR. Of the 40 transcripts, six novel transcripts were detected by quantitative real-time PCR (qRT-PCR) using seedling and VT stage samples. Five transcripts (transcripts_1, 12, 34, 35, and 40) were up-regulated in the Ki11 shoot at seedling stage, and transcripts_1, 12, and 40 were up-regulated at the re-watering stage after 12 h of drought stress. The transcripts_32 and 34 were up-regulated at the VT stage. Hence, transcript_34 possibly plays a significant role in drought tolerance during the seedling and VT stages. The transcript_32 was identified as chloramphenicol acetyltransferase (CAT) by Pfam domain analysis. The function of the other transcripts remained unknown. Further characterization of these novel transcripts in genetic regulation will be of great value for the improvement of maize production.

Keywords : *De novo,* drought stress, maize, qRT-PCR, secondary trait

Maize (*Zea mays* L.) is one of the most versatile crops in the world, serve as food, feed and industrial raw materials (Li *et al.*, 2016). In Africa, extreme yield loss over the past 10 years occurs in maize due to severe drought stress (Bänzinger *et al.*, 2006). Also, a 20-30% yield loss per year occurs in China (Gong *et al.*, 2014). Maize is especially sensitive in the seedling stage and after flowering (Bänzinger *et al.*, 2000). It is regarded to be more sensitive to drought at flowering than other rain-fed crops (Almeida *et al.*, 2013). This is due to a combination of male and female flowers, floral asynchrony, non-receptivity of the silk, tassel blasting, trapped anthers and embryo abortion (Westgate & Boyer, 1985; Lu *et al.*, 2011). Hence, drought tolerance is a very important at seedling and flowering stage.

Ki11 and Ki3 were produced as tropical lines. Ki11 was evaluated with greater drought tolerance than Ki3 (Udomprasert *et al.*, 2005). The plants have adapted by change a number of molecular, cellular, physiological and metabolic responses during drought stress (Bray, 1993; Bohnert *et al.*, 1995; Lu *et al.*, 2011). Grain yield is affected by plant height (PH), leaf senescence (LS), tassel branch (TB), anthesis-silking interval (ASI), and ears per plant (EPP) (Fuad-Hassan *et al.*, 2008). ASI among many factors was corresponded with drought tolerance data of various studies (Kim *et al.*, 2017). When drought stress was happens before flowering, it does have extreme harmful effect

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on anthesis and silk emergence (Bassetti & Westgate, 1993; Edmeades *et al.*, 2000). Drought is an important factor that affects flower growth, and it has been reported that drought stress increases ASI and negatively affects the fertilization rate, number of kernels, seed quality, and weight (Byrne *et al.*, 1995; Bolaños & Edmeades, 1996). Due to the relationship between flower synchrony and drought, the ASI is considered a secondary trait associated with tolerance and harvest index under drought (Bolaños & Edmeades, 1996; Edmeades *et al.*, 1997; Chapman & Edmeades, 1999; Ziyomo & Bernardo, 2013).

De novo sequencing technique produces millions of short sequence reads and assembling these short sequence reads without a reference genome (Shendure & Ji, 2008). In the past few years, several *de novo* sequence assembling algorithms have been developed to handle and assemble the large amount of short sequence reads to form longer fragments called contigs but choosing the appropriate assembler for paired-end or single-end data is still a challenging job (Baker, 2012; Lischer & Shimizu, 2017). The various assembly tools of short sequence reads were developed such as SOAPdenovo (Li *et al.*, 2009), ABySS (Birol *et al.*, 2009), Oases (Schulz *et al.*, 2012) and Trinity (Grabherr *et al.*, 2011). *De novo* assembly of short sequence reads is able to solve the problem of transcriptome analysis for organisms with no reference genome (Zhao *et al.*, 2011). However, the most of reads are short and contain errors and are unevenly distributed across the genome. Also, genomes contain many repetitive regions, these interfere with assembly of high quality of *de novo* genome (Treangen & Salzberg, 2012; Bradnam *et al.*, 2013; Briskine & Shimizu, 2017). Even though *de novo* assembly has some of the impediment, it is advantage to discover novel transcripts and decrease cost of sequencing.

In this study, we evaluated the secondary traits using drought sensitive line (Ki3) and drought tolerant line (Ki11). The *de novo* data of RNA-seq in Ki11 were analyzed by qRT-PCR to find novel transcripts related to the drought stress in tropical maize.

MATERIALS AND METHODS

Plant growth and drought conditions

Maize inbred lines (Ki11 and Ki3) were obtained from USDA. The plants were grown in a plastic pot 37 cm wide and 37.5 cm high (20 L) filled with soil (Plant world, Nongwoo bio) on April (Kim *et al.*, 2017). Repeated experiments were done in the year 2015, 2016, and 2017. When the tassel emergence, drought group was withheld until volumetric soil water content was around under 10% (v/v) and -1.5 (Mpa soil water potential) at 5 to 6 days after water supply. Volumetric soil water content was measured by a FDR2 (Frequency Domain Reflectometry) type sensor (WT-1000N, Mirae sensor, Korea). Soil water potential was measured by dielectric water potential sensors (MPS-6, Decagon Devices, Pullman, WA, USA). At each pot, one sensor located at a depth of 15 cm and connected to the EM50 data loggers (Decagon Devices) (Kim *et al.*, 2017). Fertilizer was applied at 20 : 15 : 15 kg/10 a of N : P_2O_5 : K_2O, respectively. Additionally, 10 kg/10 a of nitrogen was applied at 45 day after planting. When pollen shedding and extruded silk are visible, date of anthesis and silking were scored. ASI was calculated as day to silking minus day to anthesis for each plant (Buckler *et al.*, 2009). When the shedding pollen is visible, the flag leaf of each plant was collected for RNA-seq analysis and qRT-PCR in 2016.

The plants were grown in a plastic pot 240 mm wide, 380 mm length and 140 mm high (about 13 L) filled with 10 L vermiculite. We supplied 1 L water at every days, the temperature and humidity were maintained at 25°C and 40% during 16 hours with light/8 hours with dark in a growth room. Shoot and root of control group were collected for RNA isolation after removed vermiculite at 2 wk post-planting (V2 stage). Drought group was replaced from pot to paper towel after removed vermiculite for drought stress during 6 h and 12 h. Re-watering group was recovered by water supply during 24 h after 6 h and 12 h drought stress. The shoot, root, and leaf samples were conducted with three biological replicates for quantitative real-time PCR (qRT-PCR). The root samples only used 12 h drought stress. Because gene expression was highly increased in root samples of 12 h drought stress (Moon *et al.*, 2018). All of the samples were immediately frozen using liquid nitrogen and stored at deep-freezer (-80°C).

RNA isolation for qRT–PCR and RNA–seq

Total RNA was isolated from the collected samples by using the TRIzol reagent (Life technologies, GibcoBRL, leveland, OH, USA), according to the manufacturer's instructions. The quality of total RNA was assessed as nano drop (MAESTROGEN) and denatured agarose gel electrophoresis. Total RNA from the samples of each stage was used to synthesize first-strand cDNA with the ReverTra Ace® qPCR RT Master Mix and gDNA

Remover (Toyobo Co., Japan).

For RNA-seq, leaves from control and drought conditions were performed to RNA isolation with biological replication in the pollen shedding stage. Total RNA was extracted from each leaf of VT stage using the RNeasy Plant Mini Kit (Qiagen).

De novo data assembly using RNA seq

RNA quality and integrity were evaluated with a 2100 Bioanalyzer RNA Nanochip (Agilent Technologies) prior to RNA-seq. RNA-seq was performed using the Illumina HiSeq platform. The raw data of RNA-seq for each sample was analyzed by CLC genomics workbench software (Version 8.0.1, CLC Bio). The overlap settings were conducted by an insert cost of 3, length fraction of 0.8, a mismatch cost of 2, and similarity fraction of 0.8. The unmapped reads were analyzed by the following parameters: the insertion and deletion costs were set to 3, the mismatch count and maximum gap were set to 2, the length fraction and similarity parameters were set to 0.5 and 0.9, and the minimum contig length was set to 200 bp (Song *et al.*, 2018). The genes and contigs were filtered with absolute log2 fold change value greater than one. The top 40 transcripts of high expression level were selected. Forty transcripts were screened for amplification in Ki11 and Ki3 by polymerase chain reaction (PCR) and electrophoresis on 2.5% agarose gel with 1X Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer at 100 V for 30min and visualized using ethidium bromide staining.

qRT-PCR analysis of transcript for drought stress

The qRT-PCR was performed in triplicate by using the Real-Time System (BIO-RAD™, USA), and each reaction mixture contained cDNA, 10 μl of SYBR® Green TOP real qPCR 2 × PreMix (Enzynomics™, Korea), and 5 pmol of each transcript primer at a final volume of 20 μl. The qRT-PCR condition was as follows: denaturation at 95°C for 10 min, 40 cycles of 95°C for 10 s, and 72°C for 20 s; the melting curve was checked. The qRT-PCR was performed using primers for six transcripts and one reference gene (Table 1). Maize 18s rRNA was used as the reference gene (Manoli *et al.*, 2012). Relative expression level were calculated using the relative $2^{-\triangle\triangle CT}$ method by comparing to Ki3 control (Livak & Schmittgen, 2001).

RESULTS

Analysis of secondary traits under drought stress

We evaluated to secondary traits under drought stress in VT stage. Table 2 represents the results of day to pollen (DP), day to silking (DS), and ASI under control and drought conditions in 2015, 2016, and 2017.

Under drought condition, ASI of Ki3 were significantly increased that compared to control. ASI of Ki11 and Ki3 was similar every year under the control and drought conditions. DP and DS were slight different, however DS was increased every year.

Putative transcripts analysis of drought stress using *de novo* data

We assembled with *de novo* sequence using unmapped reads. A total of 182 transcripts, with a log2 fold change ratio >1.5, were found by comparing drought stress to control. The top 40 transcripts of high expression level in *de novo* analysis were selected. Forty transcripts were conducted that amplification of band were screened by PCR and agarose gel electrophoresis in Ki11 and Ki3. Of forty, 8 transcripts (transcript_1, 12, 32, 34, 35, 38, 39, and 40) were amplified by PCR.

Table 1. Primer sets of transcript and reference genes used for qRT-PCR.

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)
Transcript_1	AACTATCTCTCTCTCCCTCAGC	GGAAAAAGATGGAGTAGAGGAC	56
Transcript_12	GCGAATATATGTGTAGAAACTGC	AGACCGTAAAGAAAAATAAGCAC	56
Transcript_32	AAATGATCTGATCGATGACAAA	GTAACTTGTGACGACGACGA	57
Transcript_34	GCTGGACGTTGTTGAAGTTG	AAGAATCGTCGACGTGATCC	59
Transcript_35	AATCCATCTTGCCGTGGTAG	TTCGGCTACAGCGAGTACAA	59
Transcript_40	GAATCTGCCAGCGAGACAG	ACCGGATGAGGGGTGAGT	59
18s rRNA	CCATCCCTCCGTAGTTAGCT	CCTGTCGGCCAAGGCTATAT	59

Table 2. Day to pollen (days), day to silking (days), and ASI (days) of different maize genotypes under control and drought conditions in 2015, 2016, and 2017.

Years	Cultivars	Treatment	Day to pollen (days)	Day to silking (days)	ASI (days)
2015	Ki11	Control	91.4±1.9	92.4±3.3	1.0±2.2
		Drought	92.4±3.3	94.9±4.4	2.4±2.2
	Ki3	Control	86.4±2.4	92.9±4.7	3.4±1.6
		Drought	88.4±3.9	96.9±5.3	8.5±4.2
2016	Ki11	Control	83.4±2.3	85.9±2.3	2.5±0.8
		Drought	84.6±3.1	89.3±2.8	4.7±2.1
	Ki3	Control	79.3±1.5	81.9±2.7	2.6±1.9
		Drought	79.6±1.4	89.8±5.1	10.3±4.3
2017	Ki11	Control	81.9±3.0	83.8±3.5	1.2±1.0
		Drought	81.9±1.6	86.4±3.0	3.7±2.4
	Ki3	Control	82.9±3.0	87.9±3.6	4.4±2.0
		Drought	84.8±3.0	92.5±2.2	9.1±2.3

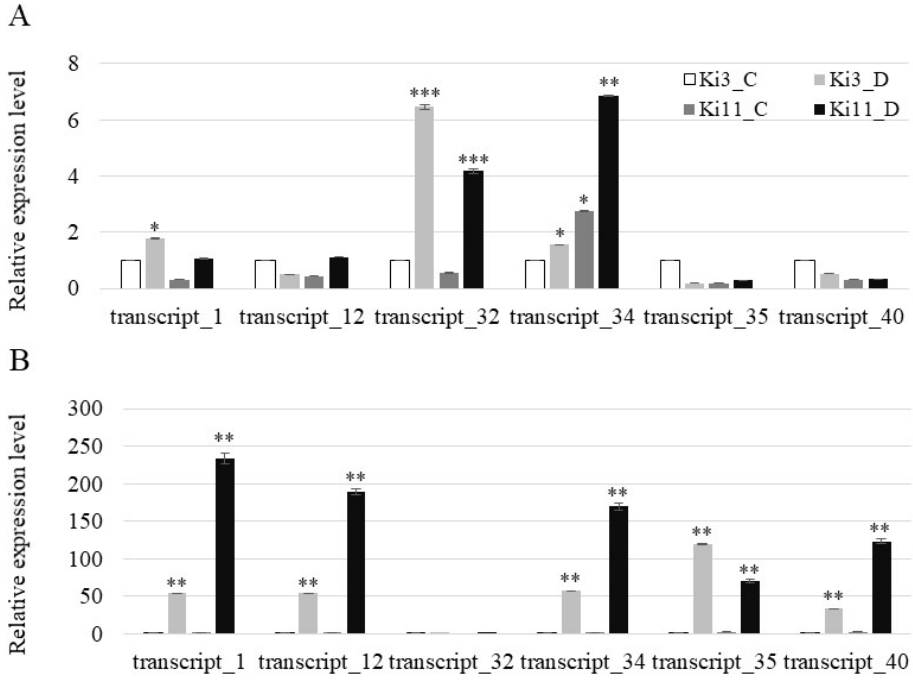

Fig. 1. Relative expression levels of Ki11 (tolerant) and Ki3 (sensitive) under control (C) and drought stress (D). Transcripts were normalized to the expression level of 18S rRNA by comparison to the Ki3 control. *Error bars* SE from three biological replicates. A) Leaf samples in VT stage. B) Root samples in seedling stage (*p<0.05, **p<0.01, and ***p<0.001, respectively).

Screening of transcripts by qRT-PCR in seedling stage and VT stage

We conducted with analysis of transcripts by qRT-PCR using seedling and VT stage samples for the three biological replicates. Eight transcripts were analyzed by qRT-PCR, 6 transcripts (transcript_1, 12, 32, 34, 35, and 40) were expressed in all samples. However, 2 transcripts (transcript_38 and 39) did not express in the most of samples.

The qRT-PCR was evaluated by comparing to Ki3 control (Figs. 1 and 2). In VT stage, transcript_32 and 34 were signi-

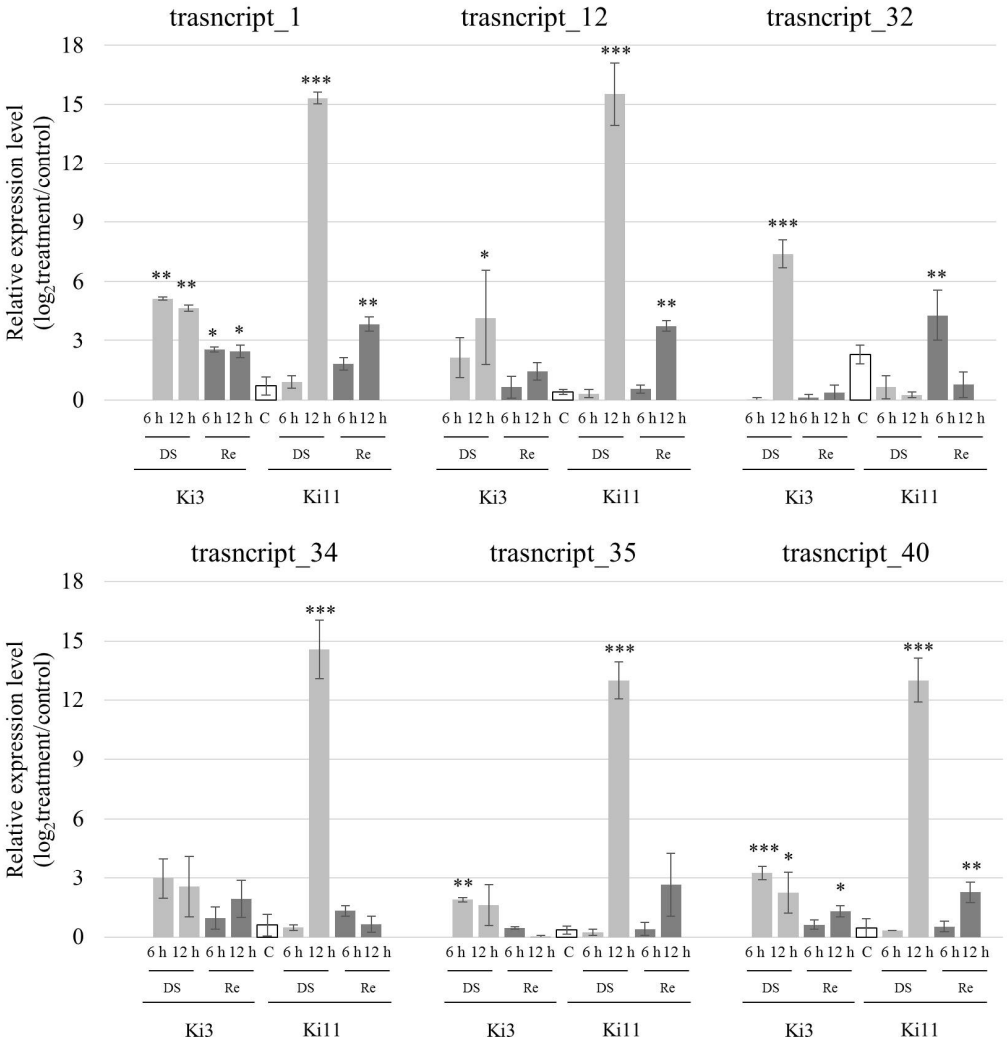

Fig. 2. Relative expression levels of Ki11 (resistant) and Ki3 (sensitive) under control (C), drought stress (6 h and 12 h), and re-watering (after 6 h and 12 h) in seedling stage. Transcripts were normalized to the expression level of 18S rRNA by comparison to the Ki3 control. Relative expression level was changed with log2. *Error bars* SE from three biological replicates (*p<0.05, **p<0.01, and ***p<0.001, respectively).

ficantly up-regulated in Ki3 and Ki11 leaf under drought condition (Fig. 1A). The transcript_1 was significantly up-regulated in Ki3 leaf. In seedling stage, Five transcripts (transcript_1, 12, 34, 35, and 40) were significantly up-regulated in Ki3 and Ki11 root under drought condition (Fig. 1B).

We conducted drought stress for 6 h and 12 h to manage the severe and extreme drought stress in seedling stage. Relative expression level of seedling stage was changed with log2. Five transcripts (transcript_1, 12, 34, 35, and 40) were significantly expressed in Ki11 shoot under 12 h drought condition (Fig. 2). The transcript_1, 12, and 40 were significantly expressed in re-watering of Ki11 after 12 h drought stress. It is possible that these transcripts were regarded with recovery response against drought stress. The transcript_1, 12, 32, 35, and 40 were significantly expressed in Ki3 shoot under drought conditions. We thought that these transcripts were responded for drought stress in VT stage, because RNA-seq samples were collected in VT stage. However relative expression level of transcripts was highly expressed in seedling stage than VT stage.

DISCUSSION

De novo sequence was produced with unknown transcripts assembly using unmapped reads to the reference genome. *De*

Table 3. Number of grains per ear (n), 100-grain weight (g), and grain yield (g/plant) of different maize genotypes under control and drought conditions in 2017.

Cultivars	Treatment	Number of grains per ear (n)	100-grain weight (g)	Grain yield (g/plant)
Ki11	Control	305.8±73.7	22.5±1.4	68.9±18.2
	Drought	277.8±35.4	22.1±1.5	61.3±8.5
Ki3	Control	295.4±43.0	22.8±1.3	67.6±12.5
	Drought	182.1±30.7	21.3±0.8	38.8±6.6

novo is powerful tool, because it is possible to sequence assembly without reference genome and background information. Hence, *de novo* sequence is consisted with not exist in reference genome. In this study, we obtained with novel six putative transcripts for drought stress. In previous studies, *de novo* assembly was conducted in wheat (Duan *et al.*, 2012), *Hevea brasiliensis* (Xia *et al.*, 2011) and *Cocos nucifera* (Fan *et al.*, 2013).

Ki11 was evaluated with greater tolerance than Ki3 under drought condition (Udomprasert *et al.*, 2005). We identified Ki11 and Ki3 using secondary traits related to the drought. These results were consistent with previous study. ASI major affected with grain yield more than the other secondary traits. According to previous study, ASI is a major secondary trait for drought index in maize (Bolaños & Edmeades, 1993; Byrne *et al.*, 1995; Bänzinger *et al.*, 2000). Drought stress at vegetative, flowering, and reproductive stage was led to reduction in grain yield (Bawa *et al.*, 2015). ASI increased from 3 to 11 days lead to 71% reduction of grain yield (Moss & Downey, 1971; Hall *et al.*, 1981). We conducted that drought tolerance estimated by various secondary traits under drought stress in VT stage. Most of phenotype results were shown that Ki11 was more tolerant than Ki3. The ASI of Ki3 increased 5.1, 7.7, and 4.7 days under drought condition in 2015, 2016, and 2017 respectively. Number of grains per ear of Ki11 and Ki3 decreased 38% and 9% respectively by drought stress (Table 3). And grain yield of Ki11 and Ki3 reduced 42.6% and 11% respectively under drought stress. Reduction of grain yield was related to inhibition of kernel development and grain filling (Shin *et al.*, 2015). Harder *et al.* (1982) reported that 100-grain weight was reduced under severe drought stress, however did not show significantly difference in this result. In this result, an increase of ASI affected decrease of fertilization rate. Hence ASI was suitable to index of drought stress in VT stage.

In the VT stage the transcript_32, and 34 were up-regulated in Ki11 and Ki3 leaf. However, transcript_1 was shown that these were significantly up-regulated in Ki3 under drought condition. The transcript_34 was regarded that down-regulated by transcription factor or other up-stream genes in Ki3.

In the seedling stage, expression level of root was similar to shoot in Ki11. While expression level of Ki3 was slightly different. Five transcripts (transcript_1, 12, 34, 35, and 40) were significantly up-regulated in root. Moon *et al.* (2018) reported that candidate genes were screened for drought stress using root of Ki11 and Ki3 under drought stress during 6 h and 12 h. The expression pattern of BU050895, CK827168, AF457983, and CF037152 was similar to 5 transcripts in root. BU050895, CK827168, AF457983, and CF037152 were found from ferredoxin-6, homeobox-leucine zipper HOX24-like, and seed maturation. BU050895 was affected with electron transport system in photosynthesis under drought stress (Lehtimäki *et al.*, 2010; Mulo, 2011). CK827168 was known with ABA-responsive element and dehydration responsive element in maize, these are decreased under drought stress (Zhao *et al.*, 2011). AF457983 and CF037152 were known as seed maturation, these are possible to relate to grain yield.

The transcript_1, 12, 34, 35, and 40 were highly expressed in Ki11 shoot under drought stress during 12 h. And transcript_1, 12, and 40 were up-regulated at the re-watering stage after 12 h drought stress.

However, transcript_1, 12, 32, 35, and 40 was up-regulated in Ki3 under drought stress. Song *et al.* (2015) reported leaf rolling scores under drought stress at the seedling stage in maize. Especially, leaf rolling is secondary traits for drought stress in maize. The leaf rolling score of Ki11 in V3 stage was evaluated with 1, 1.7, and 2.2 in moderate, severe, and extreme conditions, respectively. On the other hand, Ki3 was evaluated with 1, 2.4, and 4.2 under moderate, severe, and extreme conditions, respectively. Leaf rolling score (>4) is caused cell death and leaf dying. Ki11 had a higher seedling survival and recovery rate against severe drought stress. Hence, transcript_1, 12, 35, and 40 were possible to play a major role in drought tolerance at seedling stage. Transcript_34 was significantly up-regulated in seedling stage and VT stage of Ki11. Hence, we suggest that transcript_34 was novel gene for drought tolerance.

We predicted that function of transcripts analyzed by Pfam database. Only, transcript_32 was revealed as chloramphenicol acetyltransferase (CAT). However, the other transcripts were revealed unknown function. And we analyzed and predicted their function using blast, however all of the transcripts were not detected. These transcripts were possible that novel putative transcript relate with drought stress. These results will help to understand of transcripts for drought tolerance in maize. Further characterization of these transcripts in genetic regulation will be of great value for improvement of maize genetics.

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