Identification of Differentially Expressed cDNA in Cassava under Drought Stress Using cDNA-RAPD Approach

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Abstract

Cassava is an important carbohydrate source that provides food security and alternative renewable energy development. This plant is naturally drought tolerant, but there is a wide variation within cassava genotypes in their ability to maintain high yield and starch production under drought stress. It has been reported that cassava clones with leaf retention or stay-green trait can produce more total fresh biomass and high root dry matter compared to drought susceptible cultivars. The genetic, biochemical and molecular bases of stay-green trait, needs to be understood in order to develop drought resistant cassava cultivars since water stress limits yield and starch production. Differential Display (DD) RT-PCR is a powerful technique for analyzing differences in gene expression. The method is based on the detection of the differentially expressed cDNAs out of two or more samples. The main objective of this study was to identify differentially expressed cDNA in cassava under drought stress by employing a cDNA-RAPD approach. In this study, we used cassava genotype *Ubi Kuning* which was considered the most responsive to water insufficiency (45 days without watering). Leaf samples were collected from water-stressed and well-watered plants at day 45. Among 11 random primers, OPB03 and OPH17 have identified differentially expressed cDNA in *Ubi Kuning*. Further characterization of these PCR products of expressed cDNA under drought stress may open possibility of the development of cassava with improved drought resistance through genetic engineering and/or marker assisted selection (MAS).

Keywords: Cassava, drought, water stress, differential display, cDNA-RAPD

Introduction

Drought and high salinity are two of the most important environmental stresses that alter plant water status and severely limit plant growth, development, and productivity. Developing new crop varieties that are able to adapt environmental stress such as water shortage, water logged, extreme climate, increase salinity and other related to climate issues, would be of importance for crop yield sustainability. Environmental stresses, such as drought, salinity, cold and heat, cause adverse effects on the plant growth and development as well as plant productivity. Among the abiotic factors that have shaped and continue shaping plant evolution, water availability is the most important (Rodrigues, 2005). Cassava is one of food crops which has high prospect to be developed in marginal land as it has an ability to survive and produce higher yield in dry land than other crops such as rice and corn. Cassava as non-rice food substitution has been directly consumed widely by Indonesian people as well as raw materials in starch based industries. Because cassava tubers can be left in the soil for a couple of years, it is considered an important reserve carbohydrate source to prevent or relieve famine.

Studies of cassava drought tolerant has been started and ranged from the morphological to molecular analysis. Nasar *et al.* (2010) reported that there was positive correlation between stem color and drought response. Molecular marker aspect has extensively been studied on cassava genetic diversity using PCR-based techniques such as Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Single Nucleotide Polymorphism (SNP). RAPD technique has been used in cassava to detect markers linked to resistance to cassava anthracnose disease (Akinbo *et al.*, 2007) and amylose content variation (Sudarmonowati *et al.*, 2007).

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RAPD technique provides a cheap and rapid approach to screen a large number of accession in a breeding population. This technique could also be combined with differential expression display which is recognized as cDNA-RAPD approach. Some genes has been isolated by differential display technique such as PSBA genes encoding chlorophyll-binding proteins related to drought resistance in rice (Tyagi *et al.*, 2006; Margauthya, 2008), the genes which active in stress conditions polyethylene glycol in tea plant (Qi *et al.*, 2010), and cotton (Selvam *et al.*, 2009).

The aims of the study were to select the contrasting cassava genotypes for water insufficiency tolerance subjected to water stressed for 45 days and to identify the candidate trait marker's association with water stress condition through cDNA-RAPD approach.

Materials and Methods

Plant materials. Plant materials used for water stress treatment were six cassava genotypes of *Adira* 1, *Adira* 4, FEC 25, *Mentega* 2, *Roti*, and *Ubi Kuning*. The plants were grown in plastic pots, containing mixed peat, soil, and sand (1:1:1) and watered once a day with 550 mL of water in the morning. Some three-month-old plants (seven replicates per treatment) were used for water stress treatment.

Water stress treatment. Water stress was induced in the glasshouse by decreasing the watering volume from 550 mL (control) to mild stress (37 mL) and severe stress (no watering). Stress treatment period was 45 days and observation interval was carried out every 3 days. The parameters observed were leaf number at day 0 of water stressed induction period. The observation of leaves fallen and leaves retained number were carried out every 3 days until day-45. Plants were re-watered after 45 days stress treatment and the observation of recovery was conducted during 14 days after re-watering to identify the number of emerging new shoots.

RNA isolation, cDNA synthesis, and PCR. Total RNA was extracted from stressed water treatment and well watered (controlling) cassava young leaves using Trizol ™ isolation kit (Invitrogene) essentially following the manufacturer's protocol. The quantity of RNA was measured by a NanoDropTM 1000 (NanoDrop Technologies, USA). The quality of extracted RNA was checked using 1.5% agarose gel electrophoresis. The total RNA was reversely transcribed to cDNA. First strand cDNA synthesis was carried out by RevertAid™ H Minus First Strand cDNA synthesis kit (Fermentas) and then directly subjected to Differential Display Reverse Transcriptase Polymerase Chain Reaction (DDRT-PCR) using random primer (cDNA-RAPD). The reaction of first strand cDNA was performed in 20 µL volumes containing *Taq* DNA Polymerase 0.3 units, arbitrary 10-mer primer 0.5 µM, dNTPs 2.5 mM, and PCR buffer 1x, total RNA cDNA 3.0 µL in a thermocycler. PCR amplification products were then separated by electrophoresis on a 1.5% agarose gel.

Statistical analysis. Experiments were designed as Completely Random Design with seven replicates, Data were then subjected to analysis of variance according to the experimental design by SPSS software 13.0 version.

Results and Discussion

Selection of drought tolerant cassava

Leaf retention number or stay green leaves to be major criteria of drought resistant cassava selection. The exposure of six cassava genotypes to water stress both on mild (watering 37 mL) and severe level (no watering) for 45 days resulted in a significant increase in leaves fallen number. Control plants of six cassava genotypes tested shown value ≤ 5% of the percentage of leaves fallen number until 45 days treatment. At week 3 of drought stress period, the percentages of leaves fallen number of mild stressed water plants were ≤ 5% and >5% of genotype (Adira 1) of severe stressed water treatment. The remarkable percentages leaves falled number was observed as of week 4 (day 24 of observation interval) (Figure 1), in which almost all of genotypes tested showed ≥ 10% of percentages of leaves fallen number. During drought stress period the number of leaves per plant decreased because of a dramatic acceleration of leaf senescence and fall, and substantial decrease in leaf emergence (Catalayud, 2000). In this experiment, water deficit condition displayed significant differences in plants vigor (Figure 2). At day 45 of drought stress period most plants wilted and resulted percentages leaf retention the numberof which ranging from 27–49% out of mild water stressed plants and 19–57% out of severe

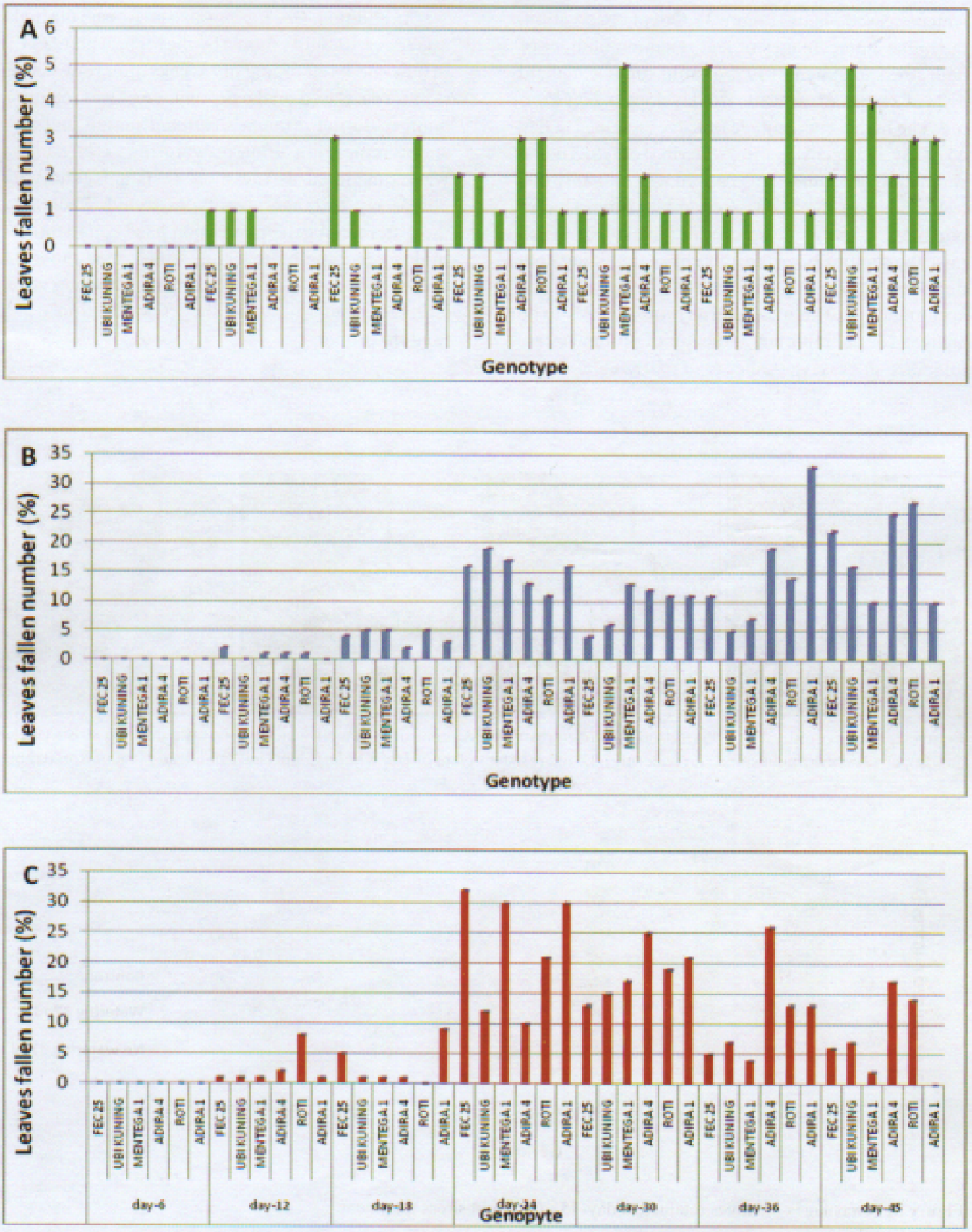

Figure 1. Percentage of leaves fallen number of six cassava genotypes during drought stress treatment. A. controlling (well-watered), B. water deficit, C. no watering.

water stressed plants (Figure 3). Out of observation, the highest percentages of leaf retention number of both stress treatments (49% of mild stress water and 57% of severe stress water) at day 45 was *Ubi Kuning*. The highest percentage of leaves retained at day 45 of severe water stress (no watering) of *Ubi Kuning* was a unique pattern. This pattern was probably due to the fact that the plants accumulate some kind of organic and inorganic solutes in the cytosol which raise the osmotic pressure, thereby maintaining both turgor and the driving gradient for water uptake. The accumulation of solutes such as proline needs to be analysed as an important indicator of drought stress tolerance in cassava (Güler *et al.*, 2012).

In addition, *Ubi Kuning* also possessed the highest recovery ability, shown by the highest number (3) of new shoots emerging in 2 weeks after re-watering (Figure 4). Based on the percentage of leaf retention number during drought treatment period and new shoots emerging after re-watering, *Ubi Kuning* was considered as the most resistant genotypes comparing to others genotypes tested. Therefore, the differential gene expression has been identified in this genotype by reversely transcribed the RNA transcript of well-watered plant and surviving water stressed plant following the PCR reaction using random primer.

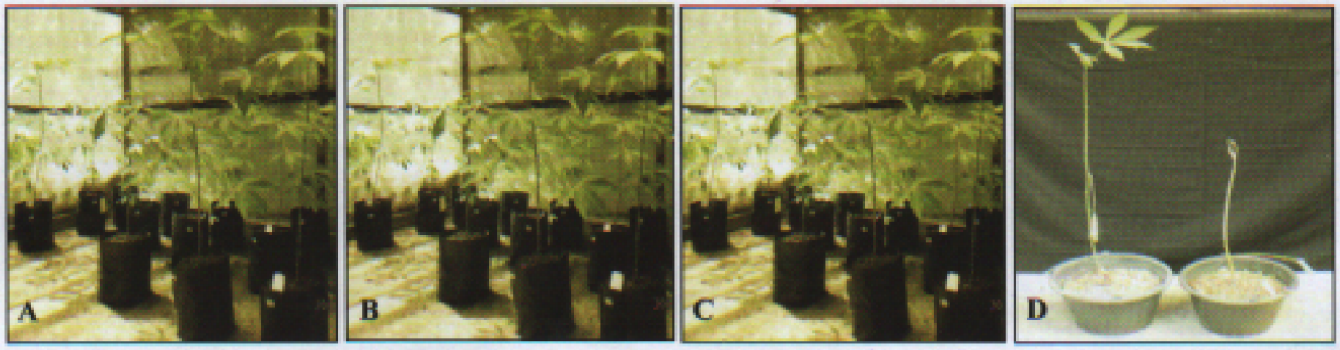

Figure 2. Plants profile at day 45 of drought stress treatment. A. controlling (well-watered), B. mild drought stress (water deficit), C. severe drought stress (no watering), D. the resistant plant of *Ubi Kuning* plant (left) and dead plant of *Roti* (right).

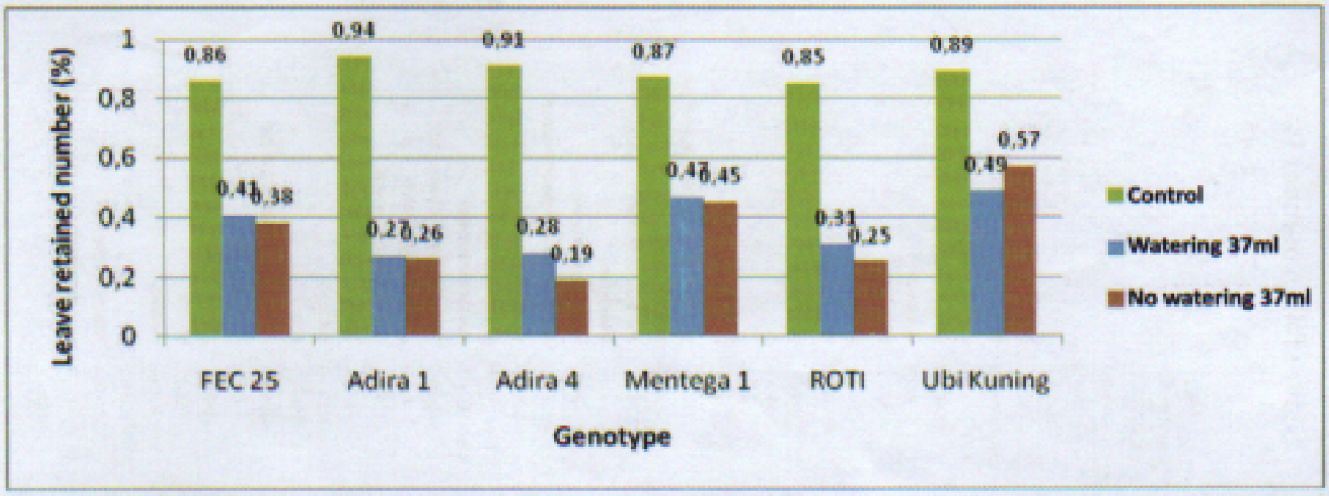

Figure 3. Percentages of leaves retained at day-45 of drought stress treatment.

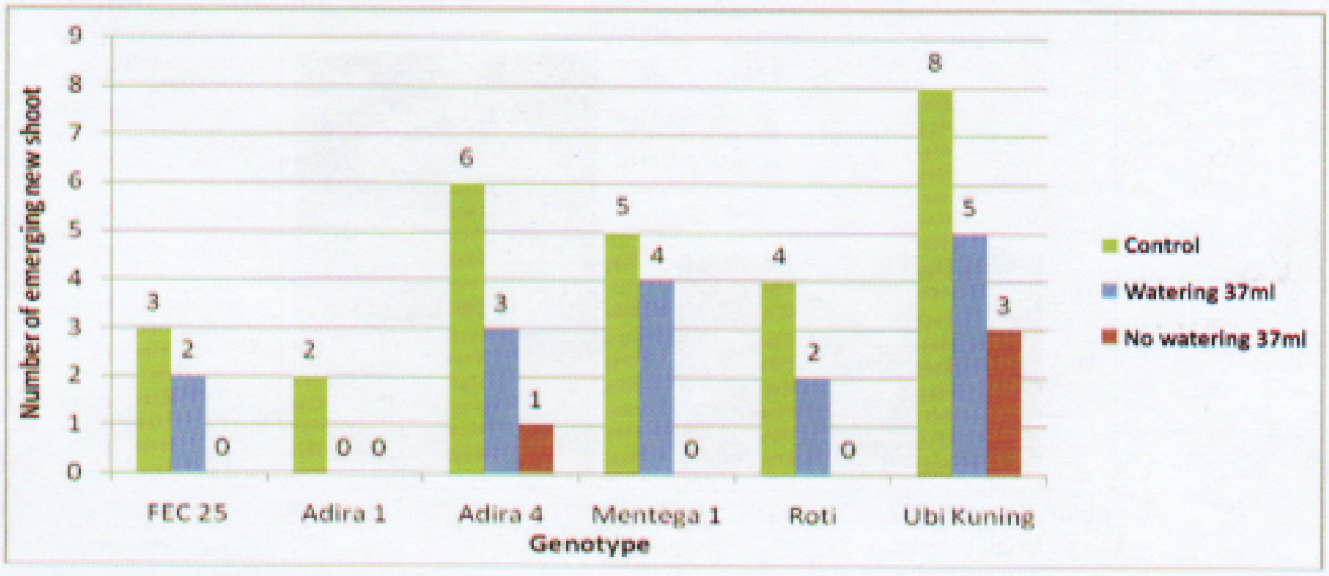

Figure 4. Number of emerging new shoot at 2 weeks after re-watering. Numbers followed by different letters are significantly different at $p<0.05$.

DNA banding patterns of amplified PCR product through cDNA RAPD approach of drought stress treatment of Ubi Kuning

Total RNA of well-watered and water stressed of *Ubi Kuning* cassava genotype have been successfully isolated using reagents Trizol™. The RNA concentration was approximately 200 ng/µL. Agarose gel electrophoresis results showed that 18S and 28S of extracted total RNA were clear and therefore that isolated extracted total RNA could be used in reverse transcription reaction (Figure 5A). In this experiment the cDNA was successfully synthesized with concentration was approximately 2,500–2,600 ng/µL (Figure 5B) and then applied in PCR amplification reaction employing random primers in order to identify differentially expressed cDNA in cassava genotype *Ubi Kuning* under severe drought stress (no watering treatment) at day 45.

Nine out of 11 primers tested could amplify the synthesized cDNA from total RNA of well- watered and water stressed of *Ubi Kuning*. The scorable band of cDNA banding pattern generated with 11 primers (OPA-10, OPB-03, OPB-13, OPE-03, OPB-20, OPE-05, OPF-04, OPH-01, OPH-17 and OPAC-16) was ranging from 1-4 (Table 1). cDNA-RAPD banding patterns generated with OPB-03, OPB-13, OPE-03, OPF-13, OPH-01, OPH-17 were polymorphic among well-watered and water stressed of *Ubi Kuning* cassava genotype. Among 6 random primers produced polymorphic band, OPB-03 and OPH-17 have identified differentially expressed cDNA in water stressed Ubi Kuning (Figure 7). Profile of DDRT-PCR:cDNA-RAPD with OPB-03 and OPH-17 showed differential expression of a gene resulted in size approximately 500 and 1,600 bp, respectively.

DDRT-PCR had increased wide interest for benefits because this technology is simple and quick and can analyse simultaneously two or more samples. This technique has been successfully applied in a variety of stressed plant research, for instance, studies about identification of drought or salinity-responsive transcripts in sunflower (Liu & Baird, 2003), peanut (Guo *et al.*, 2003), tea (Qi *et al.*, 2010), barley (Karim *et al.*, 2011) and *Erianthus arundinaceum* (Que *et al.*, 2012). The use of RAPD primer of OPA-15 reported in drought tolerant cotton shown differential expression of a gene (670 bp) of gene specific expressed during drought stress (Qi *et al.*, 2010). This research finding needs further characterization to explore the specific group or gene family of drought responsive transcript through cloning and sequencing cDNA amplified PCR product of the specific expressed bands of water stressed cassava.

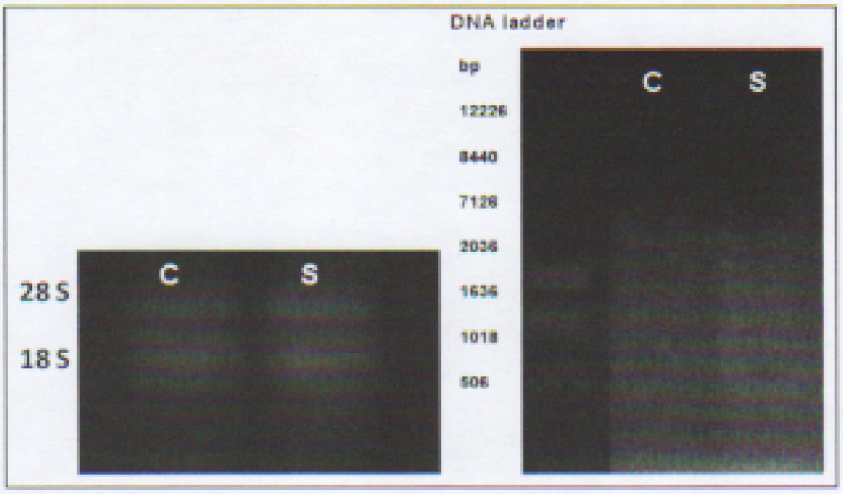

Figure 5. Total RNA (A) and cDNA synthesized (B) of well-watered (C) and no watering (S) plants.

Table 1. cDNA-RAPD Number of scorable band and specific band presented of cDNA-RAPD of well watered and no watering plants.

No.	Primers	Sequences (5' - 3')	Number of scorable band		specific band of water stress plant (no watering)
			Well watered plant	No watering plant	
1	OPA-10	GTGATCGCAG	smear	smear	-
2	OPB-03	CATCCCCCTG	1	1	+
3	OPB-13	TTCCCCCGCT	2	2	-
4	OPE-03	CCAGATGCAC	1	1	-
5	OPB-20	GGACCCTTAC	1	1	-
6	OPE-05	TCAGGGAGGT	3	3	-
7	OPF-04	GGTGATCAGG	1	1	-
8	OPF-13	GGCTGCAGAA	3	-	-
9	OPH-01	GGTCGGAGAA	2	3	-
10	OPH-17	CACTCTCCTC	2	4	+
11	OPAC-16	CCTCCTACGG	smear	smear	-

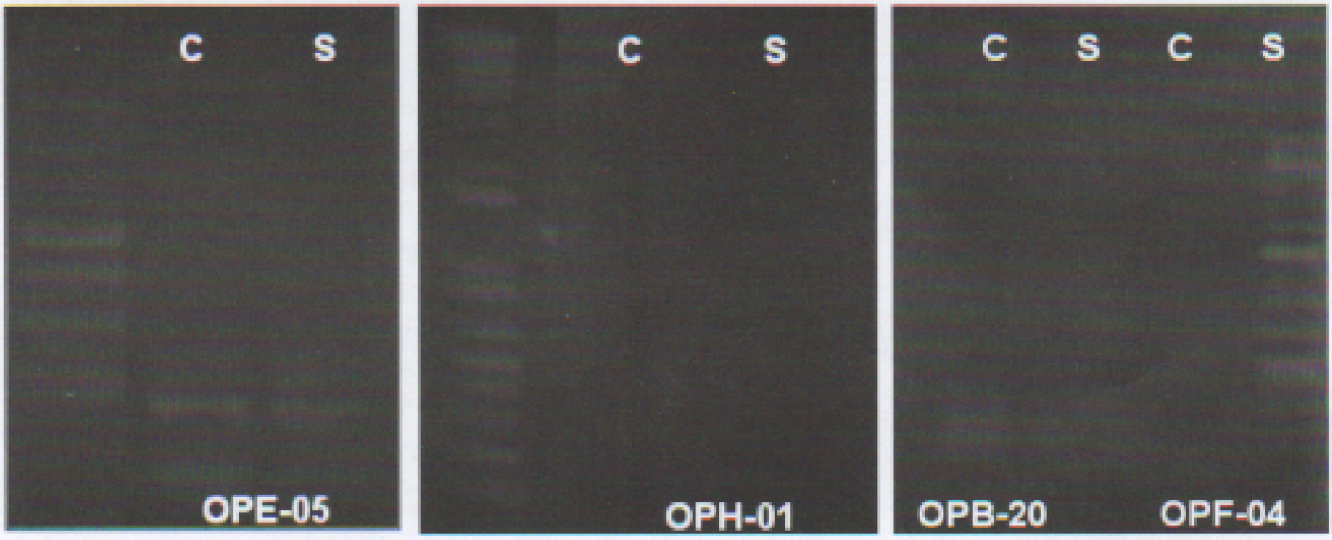

Figure 6. Profile of DDRT-PCR:cDNA-RAPD with OPE-05, OPH-01, OPB-20, OPF-04 of well-watered (C) and no watering (S) plants.

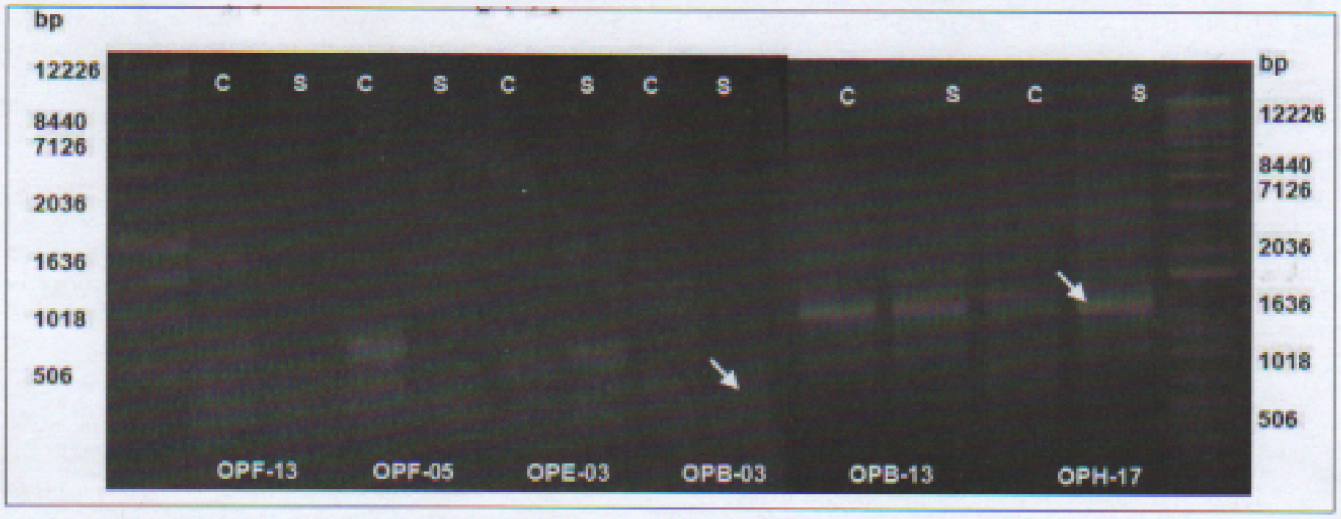

Figure 7. Profile of DDRT-PCR:cDNA-RAPD with OPF-13, OPF-05, OPE-03, OPB-03, OPB-13, and OPH-17 of well watered (C) and no watering (S) plants.

Conclusions

According to percentage leaves retention and emerging new shoots numbers, *Ubi Kuning* has been selected as the most responsive to water deficit stress compared to others genotypes tested. The differentially expressed cDNA of *Ubi Kuning* exposed to drought stress at day 45 could be identified by DDRT-PCR technique using two random primers namely OPB-03 and OPH-17 which were exhibited bands of approximately 500 bp and 1,600 bp in size. By employing a cDNA-RAPD technique it is possible in near future to identify novel drought resistance gene by comparing the gene expression profile of well-watered and stressed water plant. Recent advances in molecular techniques can intensify the efficiency of genetic improvement of crop plants for enhanced abiotic stress resistance.

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