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Isolation of Proline-Rich Protein Gene from Potato under Drought Stress

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Abstract: Drought stress is considered one of the main factors that reduce plant growth, development and production. Therefore, studying plants during drought stress is highly useful to discover the newly gained characteristics of plants. The potato is considered one of the most important crop with economic value in Egypt and over the whole world. The main target is the proline-rich protein extracted from potato plants under drought stress. A stress experiment was applied to two potato (*Solanum tuberosum* L.) cultivars Desiree and Diamante. The stress experiment was conducted by polyethylene glycol (PEG) 4000; the used water potentials were: zero (control), (PEG 2%), (PEG 4%) and (PEG 8%), after drought stress, the solutes accumulation in the two potato cultivars were determined. Increasing drought stress through elevating PEG decreased leaf area as well as shoot and root lengths. Incontorary, the chlorophyll and proline contents increased with increasing PEG treatments. The proline-rich protein gene was cloned to the pGEM-T Easy vector and was submitted to the gene bank to be used later for enhancing drought resistence in other cultivars.

1 Introduction

Potato (*Solanum tuberosum* L.) is an important food crop consumed wprldwide; however, it is sensitive towards drought stress conditions. Carbon partitioning is one of the major physiological processes affected by drought stress (Gervais et al 2021).

Plants undergoe various adaptive physiological and biochemical processes in response to abiotic stresses e.g. dehydration and excessive osmotic pressure. One of these resposes, which has drawn much attention, is the accumulation of compatible solutes such as sugars, polyols, betaines and proline (Aliche et al 2020).

The accumulated solutes can be classified into two classes; the first is nitrogen-containing compounds e.g. proline and other amino acids, polyamines and quaternary ammonium salts as well as hydroxy compounds e.g. sugars, oligosaccharides and polyhydric alcohols.

Polyethylene glycol (PEG) is reported to be able to hold water; yet it is not available to plants. The quantity of PEG required to hold water depends on its concentration and molecular weight (Van Sint Jan et al 1997). PEG is water soluble, not toxic and not easily

absorbed; these features make PEG effective in stimulating drought conditions (Al-Mullahi and Toumba 2016). To investigate how potato plants respond, *in vitro*, to drought condition, selection procedures were created utilizing a PEG solutions. During stress, the protein proline builds up in plant tissues and is considered the most prevalent osmotic regulator (Poustini et al 2007, Yadav 2010).

Plant response to heavy metal stress includes increasing proline synthesis, which lowers reactive oxygen species (ROS) by minimizing lipid peroxidation and minimizing damage to membranes (Siripornadulsil et al 2002). Different species, including bacteria, yeasts and algae, have been found to produce more proline under stressful situations (Delauney and Verma1993). Proline is known to alleviate the inhibition of various enzymes such as ascorbate-glutathione detoxification cycle enzymes (López et al 2020). It can also scaveng reactive oxygen species (ROS) and thus can reduce the adverse effects of the oxidative stress resulted from high salinity, drought, heavy metals and UV irradiation (Mohanty and Matysik 2001). The main target is the isolation of proline-rich protein gene from potato plant under drought stress using Rapid amplification of cDNA ends (RACE) to be used later for enhancing drought resistence.

2 Materials and Methods

2.1 Materials

This present work was excuted in Micro Propagation Technology Lab. At Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Giza, Egypt, during the period from 2018 to 2023. Diamante and Desiree cultivars were used in this study.

2.2 Stress treatment by using polyethylene glycol (PEG) 4000

Shoot explants were planted on a 4.42g of Murashige and Skoog (MS) media with four different concentrations of Polyethylene Glycol PEG (P); C = 0 mg, T2 = 2mg, T4 = 4mg and T8 = 8mg (Sirait and Charloq 2017). After six weeks on MS media supplemented with PEG, the following properties were measured: the average number of survival plantlets, plant height, dry weight, total proline and chlorophyll content of the leaves. Proline was determined using a UV-visible spectrophotometer (Bio-RAD) at wavelength 520 nm and expressed as mg g^{-1} dry weight (Ábrahám et al 2010).

2.3 Statistical analysis inference

Data were subjected to analysis by the SAS System, t-tests and Least Significant Difference (LSD) (Glimm et al 1997).

2.4 RT–PCR procedures

Potato leaves were used to isolate total RNA; the nano-drop (541:786ng RNA) was used to measure the quantity and purity of the isolated RNA.

A 2 g of total RNA was reverse transcribed to create the first strand of the cDNA, and the conserved portions of the proline-rich protein gene sequence predicted in the gene bank database were used to develop the degenerate primers **Table 1**.

2.5 Cloning of proline-rich protein gene into pGEM®-T Easy Vectors

The PCR product that amplified the proline-rich protein gene was purified and ligated in the pGEM-T Easy vector (**Fig 1**) by T4 DNA ligase; the insert PCR product was flanked on each side by *Eco*R1 sites at pGEM-T Easy vector (An et al 2011).

PCR, an amplified proline-rich protein gene, was purified and ligated in the pGEM-T Easy vector in a multiple cloning site that was flanked by *ECo*R1. The ligation reaction was transformed into *DH10B* competent cell and was spread onto agar plates overnight. The selected white colonies were screened by PCR using the two degenerate primers pair of proline-rich protein genes (P1 FWD, P1 RVS and P2 RVS**) Table 1**.

2.6 RLM-RACE protocol (first choice RLM-RACE kit)

Rapid amplification of cDNA ends (RACE) is a polymerase chain reaction-based technique; it is used for cloning the full-length cDNA sequences when only a partial cDNA sequence is available (Shi et al 2006).

2.7 DNase treatment for RNA

Volumes of 11 µL of RNA, 0.6 µL of DNase, 2.5 µL of 10X buffer and 10.8 µL of H_2O were added in a microfuge tube in ice. The reaction mixture was incubated at 37 °C for 30 min then 5 ml of deactivation buffer was added and incubated for 5 min at room temperature. The supernatant was collected after centrifugation at 10,000 rpm for 1.5 min (Dotti and Bonin, 2011).

Table1. Primer nucleotide sequences and concentrations of the degenerate primers for the proline rich protein coding gene (P) (P1 FWD, P1 RVS and P2 RVS) in the genebank database

Primer	Primers Sequence 5′→ 3′	concentration (nmol)
P1 FWD	ATGGGGCTTCAGTCCC TTTGTG	25
P1 RVS	CTATGGGTGAGGAAW YTTAGG	33.8
P2 RVS	TCAAGCTTGCAGTCAA TGGTC	30.8

2.8 Reverse transcription

Volumes of 7.5 µL of RNA, 1.5 µL of 10 mM dNTPs Mix and 1 µL 3' RACE adapter were placed in an RNase-free microfuge tube in ice and incubated at 70°C for 5 min, then hold in ice. Volumes of 2 µL of 10X of RT buffer, 1µL of M-MLV Reverse Transcriptase (RT) and 2 µL of nuclease-free water were added to the previous components, gently mixed, briefly spin, then incubated at 42°C for one hour and 90°C for 10 min; finally, the reaction was stored at –20°C (Bachman 2013).

2.9 Designing of RACE primer and testing them by calculating oligo web

The RACE primers were designed based on the conserved regions of the sequenced fragments. The primers were tested before synthesizing *in silico* by the Oligocalc program (Maldonado-Taipe et al 2021) **Table 2**.

2.10 PCR for 3'RLM-RACE

Two sequential rounds of the PCR were carried out; the first was conducted by the outer primer with the specific primer of proline-rich protein gene and the template was the treated cDNA for 3' end. Afterward, a volume of 3µl PCR product from the first round was used as a template for the second round. It was conducted by the inner primer with the specific primer of the proline-rich protein gene.

Table 2. Primer nucleotide sequences and concentrations of the RACE primers for the proline rich protein coding gene (F1, F2, F3, F4 and F5) on conserved regions of the sequenced fragments.

Primer	Primers Sequence 5′→ 3′	concentration (nmol)
F1	GGGCTTCAGTCCCTTTGTG	25
F2	AAGCTGAAAGAGGAATGCTAT	33.8
F3	TACACAGTGCATCAGCTGCA	30.8
F4	TACAACCCAACACCAAAGCCCA	25
F5	AGTAAAGCCACTTCCTCCTCC	25

For the first round, volumes of 1 µL of RT reaction, 5 µL of 10X PCR buffer, 4µL of dNTPs mix, 2 µL of 3' RACE gene-specific (10 µM), 2 µL of 3' RACE Outer primer and 1.25 U of thermo stable DNA polymerase (0.25 µL of 5U/µL) were assembled in an RNase-free microfuge tube in ice then the volume was completed to 50 µL of nuclease-free water. But for the second round, the same components were assembled except for adding RACE Outer primer instead of inner 3' RACE (Shi et al 2006).

2.11 Cloning of proline-rich protein gene into pGEM®-T Easy vector

A purified PCR fragment from the gene for the proline-rich protein was ligated into the pGEM-T Easy vector (An et al 2011) as depicted in **Fig 1,** and recombinant plasmids were grown in liquid media for plasmid extraction and sequencing after being transformed into *E. coli* competent cells.

2.12 Sequence analysis of the amplified fragments that potentially encode the proline-rich protein gene

The sequence analysis of the amplified fragments for the proline-rich protein gene was obtained by using two different primers, which used the forward primer located in proline-rich proteins (PRPs) and the reverse primer located in pGEM-T easy vector to decrease sequence mismatching (Zhao et al 2004).

3 Results and Discussion

3.1 Effect of PEG-induced drought stress on plant growth and developmental traits in potato plantlets

The higher concentration of PEG led to reducing leaf area, lengths of shoot and root, the number of branches and chlorophyll (Chl) content of plantlets (**Table 3**), in addition to decreasing proline content, which indicated that the potato explants were sensitive to the higher concentration of PEG.

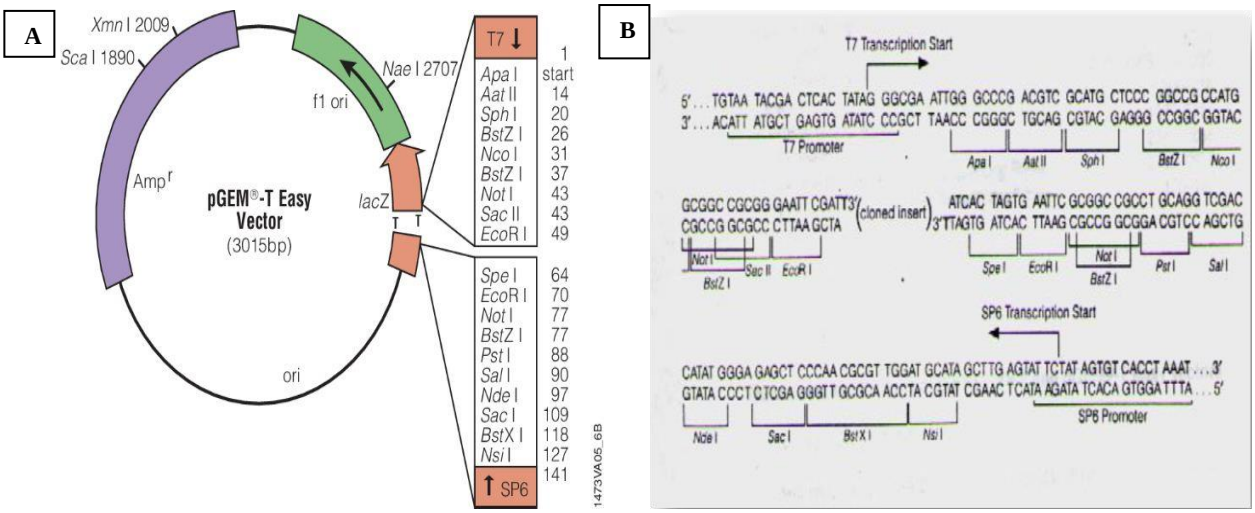

Fig 1. (A) The pGEM-T easy vector's restriction map. (B) The pGEM-T easy vector's restriction enzyme site sequence; the arrow denotes the beginning of the transcription of the T7 and SP6 RNA polymerases.

3.1.1 Survival of plantlets

After six weeks of the PEG-induced drought treatment, growth was inhibited where biomass, as well as shoot and root lengths, decreased in each of the Diamante and Desiree potato cultivars compared to their controls (0% PEG). Different concentrations of polyethylene glycol (PEG) were: C = 0 mg, T2 = 2 mg, T4 = 4mg and T8 = 8 mg as shown in **Fig 2**. PEG-induced drought treatment significantly reduced the averages of survival plantlets (**Table 3).** The results showed a significant decrease in the leaf area of T8 treatment where it was 5.443 and 3.833 cm^2 for Desiree and Diamante cultivars, respectively while in control was 8.067 and 2.300 cm^2 in Desiree and Diamante cultivars respectively. Lengths of shoots and roots significantly decreased from 29.333 (control) to 23.333 mm (T2 treatment), while Diamante cultivar showed a significant increase from 20.500 (control) to 30.667 mm of both T2 and T8 treatments. Concerning the number of branches, there were no significant differences between the control and all treatments for both Diamante and Desiree cultivars.

3.1.2 Chlorophyll content

PEG-induced drought treatment led to reducing chlorophyll (Chl) content in the leaves of both cultivars, where it was 4.167 and 1.400 µg/ml at T8 and T4 for Desiree and Diamante cultivars, respectively (**Table 3)**.

3.1.3 Proline content

In the present study, PEG-induced drought stress treatment caused proline accumulation. Free expressed proline accumulation was determined as shown in **Table 3.** In comparison to their controls, the Desiree cultivar's proline content increased with higher PEG treatment than that of the Diamante cultivar. The results showed a significant increase in the content of proline accumulation up to 0.79533 and 0.65167 µg/ml at T8 treatment compared with the control for both Desiree and Diamante cultivars, respectively.

3.1.4 The fresh and dry weight

The dry weight of the plantlets of both cultivars was lower by PEG-induced drought treatment, but no significant differences were obtained between the control and all treatments for both Diamante and Desiree cultivars.

3.2 RT-PCR amplification of proline-rich protein gene by using degenerate primer

Total RNA was isolated from Desiree plants at T8 treated with a high drought stress (PEG 8 mg). RT-PCR was applied using a cDNA of the isolated RNA from these potato plants as a template. Two degenerate primers (P1 FWD/ P1 RVS and P1 FWD/ P2 RVS) were designed based on the sequence of proline-rich protein genes and were used to amplify two fragments at 750 and 250bp in length as previously expected (**Fig 3).**

Table 3. The mean of leaf area, lengths of shoots and roots, number of branches, Chlorophyll contents in leaves , proline content and the fresh and dry weight for the PEG-induced drought treatments against the controls in the Desiree (DE) and Diamante (DI) potato cultivars.

Treatment	Leaf area (cm^2)		Lengths of shoots and roots (mm)		Number of branches		Chlorophyll (µg/ml)		Proline content (µg/ml)		Fresh weight (mg)		Dry weight (mg)	
	Desiree (DE)	Diamante (DI)	Desiree (DE)	Diamante (DI)	Desiree (DE)	Diamante (DI)	Desiree (DE)	Diamante (DI)	Desiree (DE)	Diamante (DI)	Desiree (DE)	Diamante (DI)	Desiree (DE)	Diamane (DI)
C	8.067^{a}	2.300^{c}	29.333^{ba}	20.500^{c}	2.000^{a}	2.333^{a}	6.167^{b}	25.133^{a}	0.32967^{d}	0.59067^{cb}	1.8000^{a}		0.2440^{a}	
T2	6.500^{a}	3.633^{bc}	23.333^{bc}	30.667^{a}	0.667^{a}	1.333^{a}	6.133^{b}	5.067^{b}	0.56367^{cb}	0.63633^{cb}	1.6500^{a}		0.2215^{a}	
T4	4.167^{bc}	2.567^{c}	25.000^{bac}	28.000^{ba}	0.667^{a}	2.333^{a}	4.667^{b}	1.400^{b}	0.56533^{cb}	0.54200^{c}	1.5000^{a}		0.4605^{a}	
T8	5.443^{bac}	3.833^{bc}	27.333^{bac}	30.667^{a}	2.333^{a}	1.667^{a}	4.167^{b}	4.567^{b}	0.79533^{a}	0.65167^{b}	1.7500^{a}		0.3800^{a}	
Means	6.0442^{a}	3.0833^{b}	26.250^{a}	27.458^{a}	1.4167^{a}	1.9167^{a}	5.283^{a}	9.042^{a}	0.56350^{a}	0.60517^{a}	1.3500^{b}	2.000^{a}	.3610^{a}	.2920^{a}
(LSD)	(TRT) 3.8525 (Var)1.9262		(TRT) 7.1541 (Var)3.5771		(TRT) 2.4732 (Var) 1.2366		(TRT) 10.823 (Var) 5.4117		(TRT) 0.0978 (Var)0.0489		(TRT) 0.5357 (Var) 0.3788		(TRT) 0.5781 (Var) 0.4088	

Means with the same letter are not significantly different for treatments (C = 0 mg, T2 = 2mg, T4 = 4 mg and T8 = 8 mg PEG)

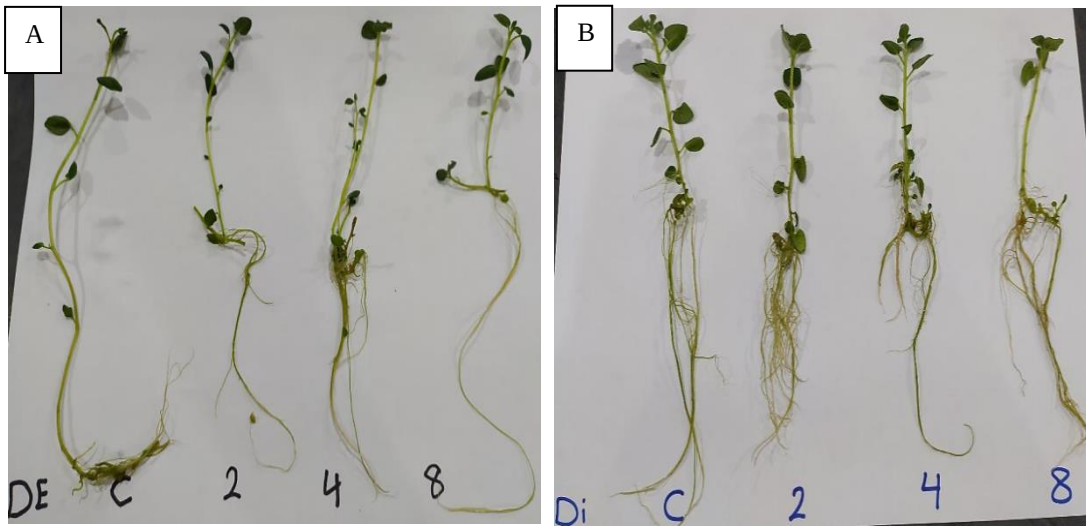

Fig 2. The lengths of shoots and roots of the PEG-induced drought treatment compared to the control in the Desiree (A) and Diamante (B) potato cultivars, respectively.Treatments are C=0mg,T2=2mg,T4=4mg and T8=8mg PEG.

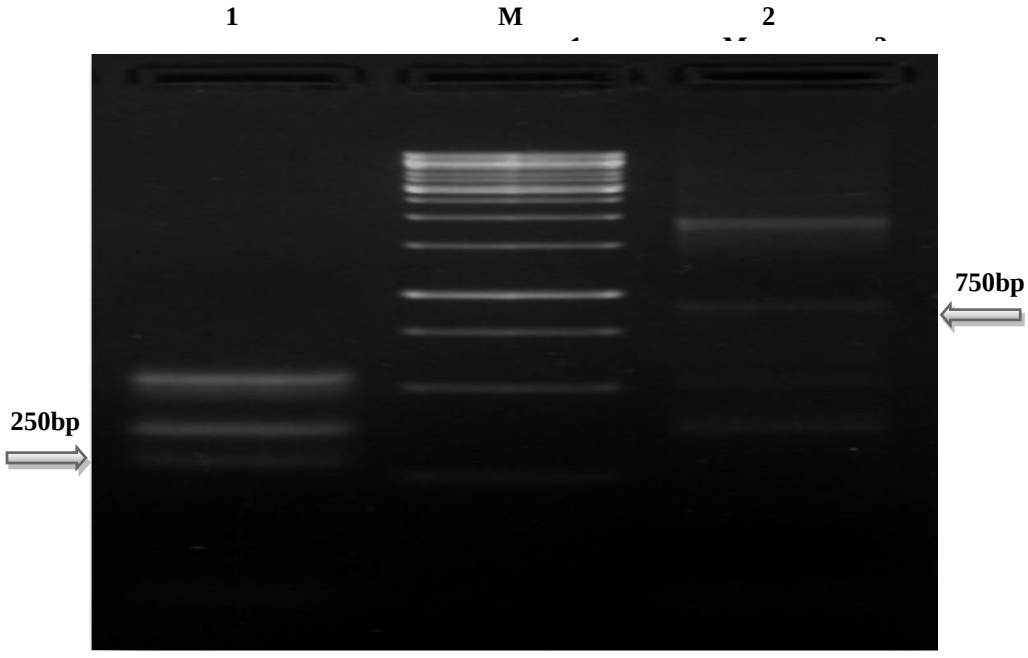

Fig 3. RT-PCR using two degenerate primers (P1 FWD/ P1 RVS and P1 FWD/ P2 RVS). Lane 1: PCR product around 250 bp in length of the first primer (P1 primer FWD/ P1 primer RVS). M: Marker 1 kb, Lane 2: PCR product around 750 bp in length of the second primer (P1 FWD/ P2 RVS)

3.3 Screening of putative colonies carrying proline-rich protein- pGEM-T easy vector

PCR screening for the white clones showed the expected result as shown in **Fig 4**. The PCR product of the second reaction (by the second primer P1 FWD/ P2 RVS) showed the expected fragment around 750bp in lanes 12:20. The expected positive clones were grown in liquid culture and the plasmids were extracted to be sequenced.

3.4 Nucleotide and amino acid sequence analysis

The nucleotide sequence of the proline-rich proteins gene (*PRPs*) from the potato cultivars was obtained by using two different primer sets; the forward was a specific primer located in the proline-rich proteins gene (PRPs) and the reverse was a universal primer located in A pGEM-T easy vector.

Additionally, the nucleotide and deduced amino acids sequence of the cloned proline-rich proteins (PRPs) gene were retrieved and compared with all published proline-rich protein gene sequences by BLAST database search.

3.5 Rapid amplification of cDNA ends (RACE) for the proline-rich protein gene using RACE primers

RACE primers were designed based on the obtained sequence of proline-rich protein fragments and used to amplify the ends of the proline gene using the PCR technique. The RACE technique idea relies on two sequential PCR (primary then nested). Each of them contains one specific primer and another universal primer (First Choice® RLM-RACE Kit).

Many primers were used, the first primer of proline-rich protein F1/outer universal RACE primer was used in the primary PCR. Then, the second primer of proline-rich protein F1/inner universal RACE primer was run with the nested primer. The result of PCR showed the expected fragments around 500:700bp in Lanes 1: 5 (**Fig 5)**. Another primer of proline-rich protein F2/outer universal RACE primer was used in the primary PCR. Then, the second primer of proline-rich protein F2/inner universal RACE primer was run with the nested primer. The result of PCR showed the expected fragments around 500:700bp in Lanes 6: 9 (**Fig 5)**. The expected fragments after nested PCR were cloned in a pGEM-T easy vector.

3.6 Screening of putative colonies carrying the 3' end of proline-rich protein- pGEM-T easy vector

PCR of amplified proline-rich protein fragment was purified, ligated in a pGEM-T easy vector and transformed into DH10B competent cells. The selected white colonies were first subjected to a screen using the PCR technique. Different primer sets as proline-rich protein F primers / proline-rich protein R primers were exploited in the screening PCR. The PCR product fragments were 850bp, 500bp, and 250bp in length as expected and shown in **Fig 6.**

3.7 Cloning selected to sequence by using RACE, T7, sP6 primers and EcoRI restriction enzyme

The putative positive vectors were confirmed using different strategies (PCR and digestion). The PCR was done by using many primers as proline-rich protein/ universal RACE primers and T7 and sP6 promoters. While the digestion was performed by the EcoR1 restriction enzyme that flanked the multi-cloning site (MCS) region in the pGEM-T Easy cloning vector **Fig 7**.

3.8 Nucleotide and amino acid sequence analysis

The confirmed vectors were subjected to sequence analysis and the 3`ends were identified. The sequence alignment was excuted by using the search facility of the National Center for Biotechnology Information (NCBI) BLAST. The identity ratio was more than 97% and 96% in cultivar *Solyntus* and variant X2 respectively with 0.0 as the e-value. The gene was submitted to the Gen Bank under accession number Bank It: ON922536.

The results of drought stress in a high concentration of PEG (8 mg) lowered the leaf area values that were 5.443 and 3.833 cm^2 for Desiree and Diamante cultivars respectively compered with that of the control (8.067 cm^2). Lengths of the shoot and root trait values was decreased in the Desiree cultivar (27.333 mm) rather than in the Diamante cultivar (30.667mm). With regard to the number of branches trait, there were no significant differences between the control and all treatments for both Diamante and Desiree cultivars. This result matched that of Sirait and Charloq (2017) who observed that PEG treatment had considerably decreased the numbers of roots, leaves and nodes, in addition to the plantlets' height and dry weight.

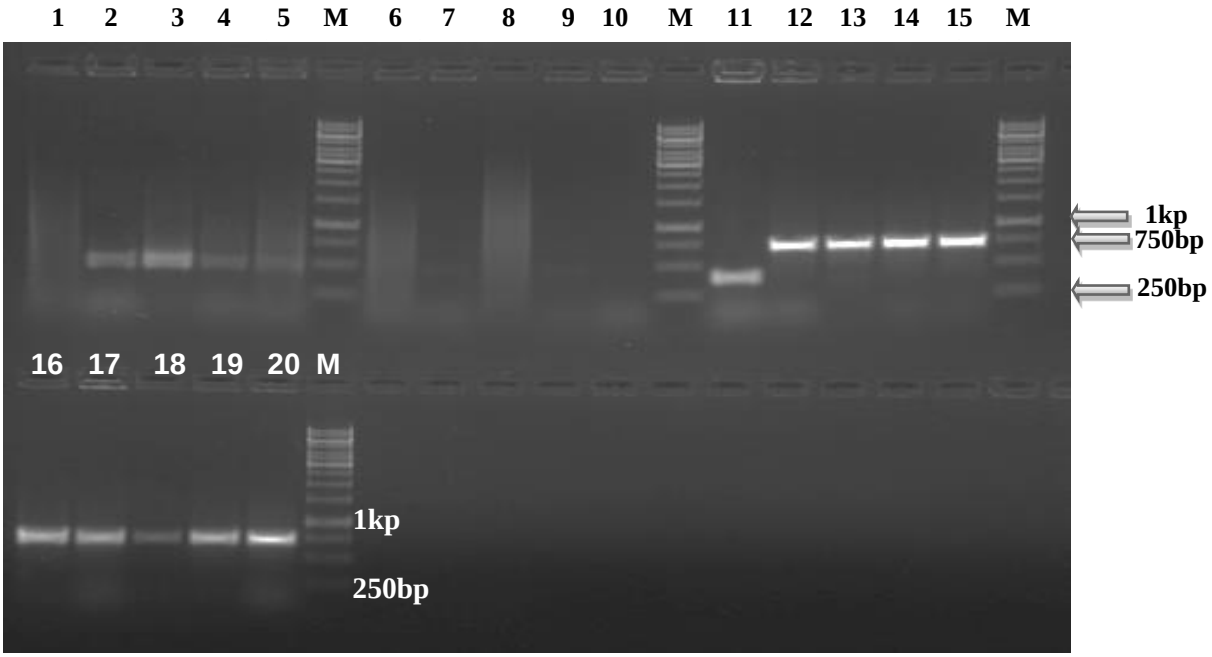

Fig 4. PCR screening using two degenerate primers (P1 FWD/ P1 RVS and P1 FWD/ P2 RVS), Lanes 11: 20 PCR products around 750 bp in length from the potential positive colonies for the second primer (P1 FWD/ P2 RVS) and M: Marker 1 kb.

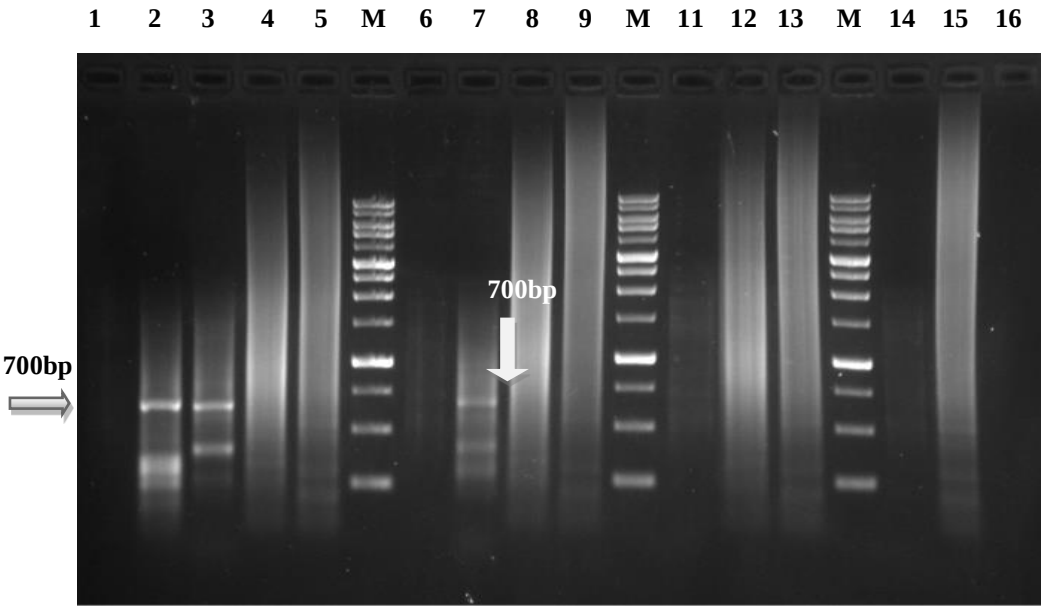

Fig 5. PCR screening using two RACE primers. Lanes 1:5 PCR products by proline-rich protein F1/ inner universal RACE primers and gave expected fragments in lane 2,3 (700bp) and (500bp), Lanes 6:9 PCR products by proline-rich protein F2/ inner universal RACE primer and gave an expected fragments in lane7 (700bp) and M: Marker 1 kb.

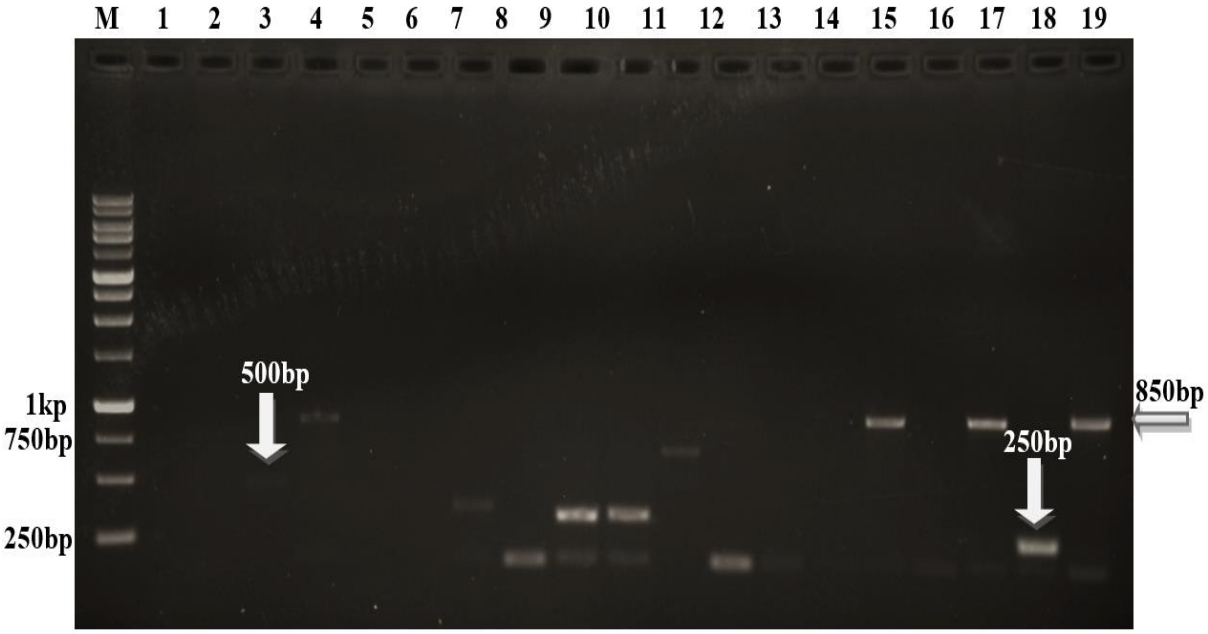

Fig 6. PCR confirmation for proline-rich protein- pGEM-T easy vector by proline-rich protein primers F and R and gave the expected fragments of 850 bp,500bp, and 250bp for PCR-amplified proline-rich protein fragments, M = Marker 1 kb GeneDirex cat no. DM010-R500.

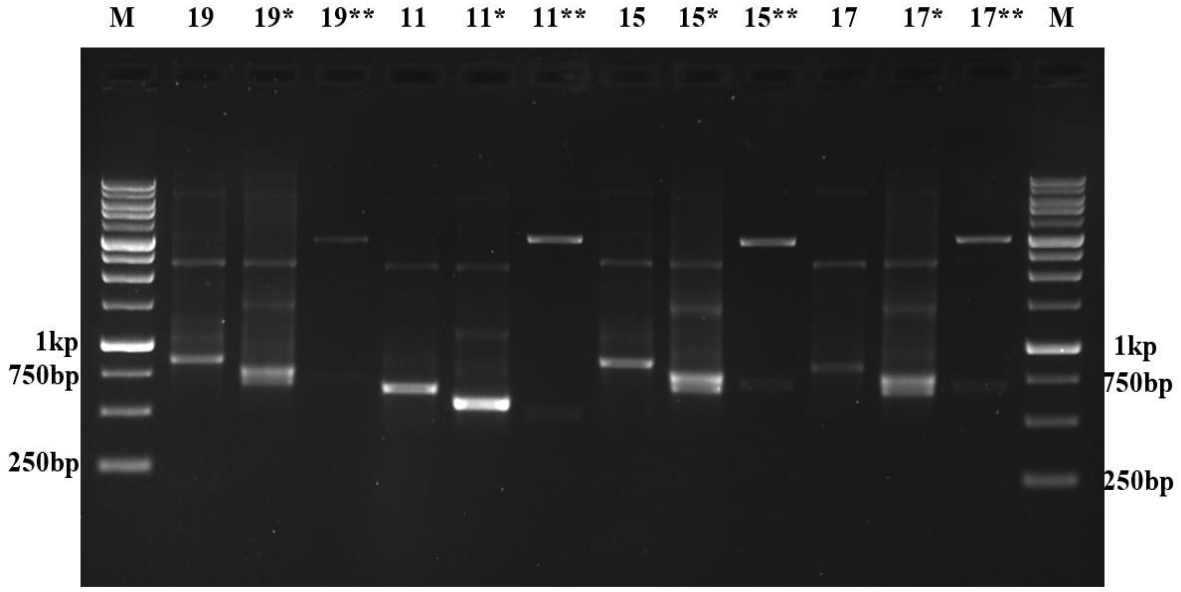

Fig 7. PCR confirmation for proline-rich protein into potato plants *via* PCR analysis by using proline-rich protein/ universal RACE primers, primers of T7, sP6 promoters (*), EcoRI restriction enzyme(**) and M:Marker 1 k.

The Chlorophyll (Chl) content trait of plantlets was decreased to 4.167 and 4.567 µg/ml for Desiree and Diamante cultivars, respectively compared with the control 6.167 and 25.133 µg/ml respectively; Xia et al (2020) suggested similar findings**.**

The content of the proline trait increased up to 0.79533 and 0.65167 µg/ml for both Desiree and Diamante cultivars compared to their controls 0.32967and 0.59067 µg/ml respectively. Similar findings in plants under saline stress were reported (Misra and Gupta 2005, Huang et al 2013, Gharsallah et al 2016).

Since Desiree cultivar showed higher increasing in proline content, it was used to isolate proline-rich protein gene. Additionally, the nucleotide sequence of the proline-rich protein gene was obtained from the Desiree plant cultivar which was treated with a high drought stress (PEG 8mg) using two degenerate primers, the RACE technique and nested PCR. This result in constitace with that of Shavalikohshori et al (2020) who used a different plant crop (*Triticum aestivum*) inoculated with *Pseudomonas* sp.

4 Conclusion

The proline-rich protein gene was isolated from Desiree cultivar which cloned in pGEM-T Easy vector and was transformed into *DH10B* strian competent cells. The isolated gene can be used later to inhance proline production and thus incease drought resistance in other cultivars.

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