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INCREASING SALINITY TOLERANCE IN THREE POTATO (*SOLANUM TUBEROSUM* L.) CULTIVARS BY TRANSFERRING mtlD GENE

ALIAKBAR ASKARI[1, 2,*] AND ASTGHIK PEPOYAN[1]

1: Laboratory of Molecular Biology and Biotechnology, Armenian State Agrarian University, Yerevan, Armenia;

2: Islamic Azad University, BAM Branch, IRI

ABSTRACT

Since one of the major reasons for yield decreasing in the world is abiotic stress, the main aim of the thesis was suggestion of new varieties of GM potato with high tolerance to salinity and water stress to Sante, Arinda and Agria potato cultivars. To achieve this aim, the following special objectives were set: i. To optimize the media for callus induction of three potatoes cultivars of Sante, Arinda and Agria; ii. To clone the mtlD gene into PBI 121 plasmid, and to transfer gen in Agrobacterium Tumefaciens LBA4404 strain; iii. To characterize and to compare the tolerance to salinity stress of new varieties of GM potatoes. We produced the transformed potato (20 plants) by transforming of mtld gene to three cultivars potato. Existence of recombinant gene in transgenic plants was confirmed by PCR analysis. For final confirmation, the transformed plants were subjected to NaCl stress, then physiological parameters were compared between transgenic and non- transgenic potatoes. The transgenic potatoes (three cultivars) showed the different amounts of tolerance to salinity stress because these plants produced mannitol which increased osmotic pressure in salinity stress and caused plant be able to absorb water from saline soil .Therefore we can conclude that mtlD gene was expressed in transgenic potatoes.

Key Words: Transgenic Potato, mtlD Gene, Salinity Stress

INTRODUCTION

Economically, potato is one of the world's most important tuber crops, belonging to the solanaceae family. In Iran potato is cultivated over an area of 200000 ha. Potato (*Solanum tuberosum* L.) is the starchy, tuberous crop of solanaceae family and the world 'fourth largest food crop after rice, wheat and corn **(Bajaj, 1987)**. Genetically modified organisms (gmos) can be defined as organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally. The Technology is often called “modern biotechnology” or “gene technology”, sometimes also “recombinant DNA technology ” or “ genetic engineering” . It allows to select individual genes to be transferred from one organism into another, also between non-related species. For the production of GMO plants (genetically modified plant), new gene was transferred to a plant. There are two main methods for transferring the gene into plants cells and tissues. The "Gene Gun" method (also known as microprojectile bombardment or biolistics). The Agrobacterium method, Transformation by Agrobacterium has been successfully practiced in dicots **(Bottino *et al.*, 1989)**. At first, this method was introduced as a simple and general method for transferring genes into plants cells **(Horsch *et al.*, 1985)**. With transferring mtld gene which causes producing mannitol, to plant by plasmid agrobacter, increases osmotic pressure in potato. Due to this, resistance increases to salinity stress.

Abiotic stresses, such as drought, salinity, high temperatures, frost, chemical toxicity and oxidative stresses are serious threats to agriculture and result in the deterioration of the environment **(Bartels and Sunka, 2005)**. They are the primary causes of crop loss worldwide. Abiotic stresses adversely affect growth and productivity and trigger a series of morphological, physiological, biochemical and molecular changes in plants **(Bhatnagar-mathur et al., 2008)**. Drought, temperature extremes, and saline soils are the most common abiotic stresses that plants encounter. Globally, approximately 22% of the agricultural land is saline **(FAO 2004)**, and areas under drought are already expanding and this is expected to increase further **(Burke *et al*. 2006)**. Drought and salinity may cause serious salinization of more than 50% of all arable lands by the year 2050. Besides, world population is growing at an annual rate of about 1.5%, and expected to be over 10 billion by 2050 **(Bhatnagar-mathur *et al*., 2008)**. Plant genetic engineering strategies for Abiotic stress tolerance rely on the expression of genes that are involved in one of the following approaches:

• Signaling and regulatory pathways **(Seki *et al*., 2003 & Shinozaki** etal**., 2003)**.

• Genes that encode proteins conferring stress tolerance **(Wang *et al*., 2004)**

• Enzymes present in pathways leading to the synthesis of functional and structural metabolites.

Sugar and sugar alcohols such as mannitol, fructans and sorbitol occur in as many as 50 families of higher plants. Although their physiological functions are not clear, accumulation of these compounds often occurs as a result of water stress. There are many reports on using sugar alcohols for stress tolerance via genetic transformation by modifying the responsible enzymes in their biosynthesis pathway **(Tarczynski *et al*., 1992)**.

Tarczynski in 1992, studied on expression of a bacterial mtld gene in transgenic tobacco leads to production and accumulation of mannitol. Mannitol concentration exceeded 6 Mmol/g (fresh weight) in the leaves and in the roots of some transform ants, whereas this sugar alcohol was not detected in these organs of wild- type tobacco plants or untransformed tobacco plants that underwent the same regeneration scheme. Plants containing mannitol had an increased ability to tolerate high salinity. **Abebe in 2003**, showed that expression of the mtld gene for the biosynthesis of mannitol in wheat improves tolerance to water stress and salinity.

In this research for the production of transgenic potato, three steps were performed including **1. Callus induction 2. Gene transformation 3. Study of GMO potato features**. The aim of this research was the production of transgenic potato for increasing its tolerance to salinity stress.

METHODOLOGY

Plant material: The tubers of three cultivars: Sante, Arinda and Agria, of *Solanum tuberosum L.* were kindly provided by the Agricultural Research Center of Bam (Iran).

Plasmid: The Plasmid PBI121 was used for transferring gene to potato plant as a vector. The T-DNA region of this vector contains the right border, expression cassettes for a neomycin phosphotransferase II (NPTII) selection marker and a beta-glucuronidase (GUS) reporter gene, and the left border. The non-T-DNA region (8565 bp) was constructed according to the Bin 19 vector.

Callus induction and disinfection.

The potato tubers were planted in pots (25*20cm) filled with a mixture of peat and vermiculated (3:1). After enough growth, stems with 3-4 leaf buds were sampled. These explants were rinsed in 70% alcohol for 1-2 minutes in a sterilized Erlenmeyer flask. Then they were sterilized with 10% commercial Clorox (NaOCl) for 15 minutes, and then rinsed 3 times with sterile distilled water. All the tools required for dissection were previously sterilized. Disinfected leafs buds were dissected out into small pieces (0.5 - 0.8 mm) and placed separately in some culture bottles containing 10 ml of MS medium with various growth regulators. IAA (Indole- 3-Acetic Acid), kinetin and 2,4- D were also added to the Murashige and Skoog medium in different concentrations and combinations. The pH of the medium was adjusted to 5.7 with either 1 N KOH or 1 N HCl. The culture media were autoclaved at 151b/in2 with 121°C for 15 minutes, then maintained at 27±2°C in the culture room.

All operations were carried out in a laminar airflow cabinet. The culture bottles containing the explants were maintained in the laboratory at 27±2˚C. They were exposed to artificial illumination of 2000-3000 lux by placing them at 25-30 cm below fluorescent light for sixteen hours every day. Mediums were including MS media supplemented with 1 mg/l 2,4-D, 2 mg/l

2,4-D, 2 mg/l kinetin,3 mg/l kinetin, 1 mg/l IAA and 2 mg/l IAA. Callus weight and callus size were measured. After 4-5 weeks, calluses were harvested and callus size and weight were recorded.

Gene Transformation

Tubers of the three potatoes lines (+mtlD and -mtlD) were placed in 5% sodium hypochlorite solution for 15 minutes, and then they were washed by distilled water three times for 5 minutes. These sterilized tubers were planted in pots, which filled with disinfected soil, by water vapor. After 7 days the pieces of terminal meristem (leaf buds) were isolated and were cultured on prelature containing basal MS semi-solid medium with 1.4% agar, supplemented with 2% sucrose, and 2,4-D (2mg/l) for callus induction. The cultures were maintained at 20-22°C under cool, white fluorescent lamps with 16h photoperiod.

To produce a transgenic potato plant resistant to salinity stress, mannitol, 1-phosphate dehydrogenase gene (mtlD, E.C.1.1.1.17) was used. The mtlD gene was isolated from pCabmtlD plasmid (*E. coli*) and then was cloned in PBI121 plasmid (LBA4404). The promoters that have been most commonly used in the production of abiotic stress tolerant plants is the CaMV 35S **(Romero *et al*., 1997)**. This vector was inserted into (*Agrobacterium tumefaciens*) which was used to produce transformed potato plant and was stored in 28°C temperature. The freeze-thaw method was used for replacement of recombinant binary vector PBI121 into (*Agrobacterium tumefaciens*) strains LBA4404. The *Agrobacterium tumefaciens* was inserted in LB (Lysogeny broth) medium and was centrifuged for 3 h in (4000-5000rpm) then lower part of solution was inserted in ½ MS medium. The most common growth media for bacteria are nutrient broths (liquid nutrient medium) or LB medium (Lysogeny Broth). The explants (leaf bud or terminal meristem) which had inserted in precultur were incubated with (*Agrobacterium tumefaciens*) containing the 35S-mtlD gene for 5 min **(Horsch *et al*., 1985)**. After co-cultivation for 2-3 days, the explants were transferred to liquid medium containing 250 mg/l cefotaxim and 100mg/l kanamycin to select transformed cells and was checked by PCR technique. These transformed explants were placed in callus induction culture then were transferred to MS medium, supplemented with shoot induction medium (MS+2mg/l BAP+0.2mg/l NAA) then shoots (5-6mm length) were transformed to root induction medium (IAA, 1mg/l) for organogenesis. For disinfecting all hormones and antibiotics syringe filter (0.22µm) method was used. 2, 4-D hormone was dissolved in methanol but other hormones and antibiotics were dissolved in distilled water.

Formation of GM varieties of potato, and its characterization: We produced the transformed potato. Only 20 lines were positive for mtlD gene from 100 lines in PCR. The mtlD gene produce mannitol and had been isolated from *Escherichia coli* then this gene had been cloned in *Agrobacterium tumefaciens* (LBA 4404) by use of cacl2 and liquid nitrogen by freeze –thaw method. Existence of recombinant gene in transgenic plants was confirmed by PCR analysis. Total DNA was extracted from plant tissues with cetyl (hexadecyl) trimethyl ammonium bromide (CTAB), essentially as described by Wagner in 1987. Inserted DNA was detected by PCR. For final confirmation, the transformed plants were subjected to NaCl stress for 30 days. Therefor the transgenic plants were sown in pots and experiment was conducted as a factorial arrangement using a Randomized Complete design with four replications. The treatments were the different amounts of salt solutions (0, 50, 100, 150 mol/m3 NaCl) for 30 days. The non-transgenic plants were used as controls. Growth was measured at the end of the stress period. For plants, fresh weight, dry weight, total tuber weight, tuber number, root weight, harvest index and shoot weight were measured. Physiological measurements were done two week after the salt stress treatments and the data was analyzed by SPSS software.

RESULTS AND DISCUSSION

Callus growth of potato cultivars: The presented data in **Table 1-3** demonstrate that number of the treatments induced the callus formation, while the others did not induce it, and at all, during the first week of incubation, little morphological differentiation and slight increase in tissue volume of calluses was observed. The size and weight of callus are also showed in **Table 1-3**. It is obvious, that the 2 mg/l of IAA brings to the decrease of callus in size and weight. At the same time, as show the results of **Table 1-3**, the different hormones' combination showed the different results for callus size and callus weight in three potato cultivars. The comparison of these effects declared that 2,4-D hormones in 2 mg/l concentration and Kinetin in 3 mg/l had more influence on both callus size and callus weight. In addition, the Arinda cultivar showed the more callus induction than two other cultivars **(Table 1-3)**.

Characterizations and comparisons of new formatted transgenic potato cultivars

Data analysis of all growth indicators showed the negative influence of salinity stress (different NaCl concentration) on two potato lines of *Arinda* cultivar (+mtlD and – mtlD). Considerably, the recorded growth indicators reduction in +mtlD (GMO potato) line was recorded more than – mtlD lines. In –mtlD plants, salt stress reduced fresh weight by 40% in 50 mM NaCl , 65% in 100 mM NaCl , 87% in 150 mM NaCl while in +mtlD potatoes salt stress decreased fresh weight by 20% in 50 mM NaCl , 44% in 100 mM NaCl , 55% in 150 mM NaCl . In –mtlD plants, salt stress reduced dry weight by 45% in 50 mM NaCl , 67% in 100 mM NaCl , 81% in 150 mM NaCl but in +mtlD potatoes salt stress decreased dry weight by 19% in 50 mM NaCl , 37% in 100 mM NaCl , 45% in 150 mM NaCl **(Table 4)**.

The results of investigation on cv. *Agria* potato plant were presented in **Table 5** The comparative analysis showed that there is significant difference between the four salinity stress treatments (0,50, 150, 200 mol/cm^3 NaCl) and there is significant difference between the two mtlD treatments (+mtlD, -mtlD) in all growth indicators, by recorded probability alpha= 0.01. Data analysis of all growth parameters indicated the negative effect of salinity stress (different NaCl concentration) on two potato lines (+mtlD and – mtlD). Noticeably, the recorded growth indicators reduction in +mtlD (GMO potato) line was recorded more than – mtlD lines. In –mtlD plants, salt stress reduced fresh weight by 39% in 50 mol/cm3 NaCl , 61% in 100 mol/cm3 NaCl , 73% in 150 mol/cm3 NaCl while in +mtlD potatoes salt stress decreased fresh weight by 28% in 50 mol/cm3 NaCl , 44% in 100 mol/cm3 NaCl , 56% in 150 mol/cm3 NaCl. In –mtlD plants, salt stress reduced dry weight by 32% in 50 mol/cm3 NaCl, 55% in 100 mol/cm3 NaCl , 73% in 150 mol/cm3 NaCl but in +mtlD potatoes salt stress decreased dry weight by 14% in 50 mol/cm3 NaCl, 30% in 100 mol/cm3 NaCl , 35% in 150 mol/cm3 NaCl **(Table 5)**.

In addition, data analysis of all growth indicators in *Sante* cultivar showed the negative effect of salinity stress (different NaCl concentration) on two potato lines (+mtlD and – mtlD). Considerably, the recorded growth indicators reduction in +mtlD (GMO potato) line was recorded more than – mtlD lines **(Table 6)**.

The properties of produced genetically modified potatoes at next stage were evaluated. For this aim, the 2 ways were applied, **1. PCR 2. Physiological measurement of transgenic plants**.

The effect of salinity stress on growth of transgenic and non-transgenic three cultivars of potatoes is presented in **Table 4-6**. The results of these tables indicate that there is significant difference between four salinity stress treatments (0, 50, 150, 200 mM NaCl), and there is significant difference between two mtlD treatments (+mtlD, -mtlD) in all growth indicators, by recorded probability alpha= 0.01. Considerably, interactions recorded significant differences among all treatments.

Table 1: The growth of callus of Sante and Arinda potato cultivars.

Treatments	Hormones concentration (mg/l)			Callus formation*		Callus size (cm3)		Callus color		Callus weight (gram)	
	2,4-D	IAA	Kin	Sante	Arinda	Sante	Arinda	Sante	Arinda	Sante	Arinda
1.	1			+	+	2.4	2.7	White	White	1.8	2.2
2.	2			++	+++	3.2	3.9	Green	Light green	2.2	4.8
3.		1		+	+	2.3	1.5	White	White	1.1	2.7
4.		2		+	++	1.4	1.6	White	White	1.1	1.3
5.			2	+	++	1.8	3.4	Light green	Light green	1.9	1.6
6.			3	++	+++	3.2	3.5	White	Greenis h white	1.7	3.2

*Growth expression: + Low growth; ++ - Normal growth; +++ Good growth; 2,4-D- dichlorophenoxyacetic acid; IAA- indole- 3- acetic acid; Kin- kinetin

Table 2: The growth of callus (callus size, callus weight) of tow potato cultivars (*Agria and Sante*)

Treatments	Hormones concentration (mg/l)			Callus formation*		Callus size (cm^3)		Callus color		Callus weight (gram)	
	2,4-D	IAA	Kin	Sante	Arinda	Sante	Arinda	Sante	Arinda	Sante	Arinda
1	1			+	+	3.0	3.6	Greenish white	white	2.2	3.3
2	2			+	++	3.8	4.3	green	Light green	3.4	4.2
3		1		+	+	1.8	1.7	_	white	1.6	2.8
4		2		+	++	2.3	2.5	white	white	2.0	3.0
5			2	+	++	2.9	3.2	Light green	Greenis h white	2.2	2.7
6			3	+	++	3.4	3.5	Light green	Greenis h white	3.0	3.8

Growth expression: + Low growth*;
Normal growth - ++
Good growth - +++
.D- dichlorophenoxyacetic acid; IAA- indole- 3- acetic acid; Kin- kinetin-2,4

Table 3: The growth of callus (callus size, callus weight) of tow potato cultivars (*Arinda and Agria*)

Treatments	Hormones concentration (mg/l)			Callus formation*		Callus size (cm^3)		Callus color		Callus weight (gram)	
	2,4-D	IAA	Kin	Sante	Arinda	Sante	Arinda	Sante	Arinda	Sante	Arinda
1.	1			+	++	3.0	3.0	Green	Greenish White	2.9	3.6
2.	2			++	+++	3.3	3.5	Green	Light green	3.7	4.0
3.		1		+	+	1.5	1.0	White	White	1.8	2.0
4.		2		+	+	2.0	2.0	Light green	White	2.5	2.5
5.			2	+	++	2.0	2.5	Light green	white	2.5	3.0
6.			3	+	++	2.5	3.0	White	white	3.0	3.5

Growth expression: + Low growth*
Normal growth - ++
Good growth - +++
.D- dichlorophenoxyacetic acid; IAA- indole- 3- acetic acid; Kin- kinetin-2,4

NaCl *; Plant*; Fresh weight (g); Dry weight (g); Height (cm); Number of tubers; Total weight of tubers (g); Harvest Index; Shoot weight (g)