ENHANCEMENT OF DROUGHT TOLERANCE IN RICE (*Oryza sativa* L.) USING OXO-PHYTODIENOATE REDUCTASE 7 (*OPR7*) GENE

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The Egyptian fertile land comprises 4% only from the total Egyptian land area. This fertile area is found only in the Delta and Nile valley. The remaining 69% of the total Egyptian lands ($1x10^6$ km^2) are arid lands (El-Ramady *et al.,* 2013). Although Egypt has one of the longest rivers in the world, limited water resources are considered as one of the major challenges that face agriculture development and land reclamation. Agricultural as well as all Egyptian water requirements secured from three different water resources; the Nile River, sea water distillation and groundwater. These resources secure about 76.7%, 0.08%, and 28%, respectively (CAPMAS, 2009). On the other hand, global climate changes lead to change in the land nature toward aridity (Handmer *et al.,* 2012) which makes the situation of water availability more vulnerable. These water deficit and drought challenges hinder the Egyptian agricultural policy which aims to increase the overall production by at least 70% in the next 30 years in order to cope with the steady increase in the population census and to secure the necessary food (Aune, 2012). Therefore, development of drought tolerant plants is one of the challenging approaches which required interference in the plant's physiological mechanisms and genetic control (Farooq *et al.,* 2009).

The phytohormones jasmonates (JAS) have been shown to participate in plant response to abiotic stress through integration with key factors in signal transduction pathways. JAS are a group of oxylipin compounds occurring ubiquitous-

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ly in the plant kingdom that can mediate plant physiology and alleviate environmental perturbations by responding to the developmental and environmental signals (Li *et al.,* 2011). Flavoenzymes are considered as key protein elements that participate in the biosynthesis of jasmonates and play an important role in drought stress in rice (Tani *et al.,* 2008). Flavoenzymes are flavin mononucleotide-dependent oxidoreductases which are coded by oxo-phytodienoate reductases (*OPRs*) multigene family in many species (Li *et al.,* 2011). In rice genome, the RAP-DB and TIGR databases identified 13 *OPR* homologue genes. One of these identified homologues codes for *OPR7* which catalyzes the reduction of 12-oxo-phytodienoic acid (OPDA) in the octadecanoid pathway for the biosynthesis of Jasmonic acid (JA) (Li *et al.,* 2011). It has been proven that *OPRs* could enhance plant tolerance against different abiotic stresses. In an attempt to enhance the tolerance of tomato plants against salt stress, *AtOPR1* gene was transformed into two tomato (*Solanum Lycopersicum*) genotypes. The tomato transgenic lines showed an increasing in their salt tolerance (El Nagar, 2013). This study and similar other studies have opened the way for the use of *ORPs* genes and other related genes in improving the ability of some important plants to resist the stresses.

As a cereal grain, rice is one of the main important food items in most countries of the world, especially developing countries. The production of rice has to be increased by 70% by 2050 to meet the demands of the global population and secure food especially that the population of the world estimated to be 9.5 billion by 2050, (Bakshi and Dewan, 2013). To reach this goal, it is a must to find solutions to face the severe loss of rice yield because of different stresses especially drought. Rice has a semi-aquatic phylogenetic origin; its production requires warm and wet environment and an efficient water intensive system (Wassmann *et al.,* 2009). Therefore, water shortage and drought stress are the main threats to rice production (Mukamuhirwa, 2015). In Egypt, rice is cultivated in the Delta region in an area exceed one million Fadden with productivity about 3.5 tons per Fadden. Rate of rice consumption in Egypt is about 35-40 kg/capita/annum. The annual production rate is about 5.5 million tons which has to be increased to meet the demands of the Egyptian population (Elmoghazy and Elshenawy, 2018).

It was difficult to develop drought tolerant cultivars by using classical approaches due to the complexity of drought tolerance mechanisms, diversity of the genotypes, interaction of environmental factors and the rareness of having an appropriate screening technique (Verulkar *et al.,* 2010). As mentioned before, development of genetically modified cultivars could significantly improve its stress tolerance and this requires genetic resources and gene mining process (El Nagar, 2013; McCouch, 2013). Although, hundreds of rice genes have been sequenced and there are many genes reported to have a vital role in gaining drought tolerance in rice,

there is a clear limitation in the production and development of drought tolerant rice varieties (Todaka *et al.,* 2015). Despite of these difficulties, it is necessary to enhance drought tolerant in rice cultivars as well as a well organized irrigation system to help in facing the drought and water deficiency threats and to increase rice productivity (Serraj *et al.,* 2011). Development of genetically modified rice becomes routine work after establishing the rice transformation systems by *Agrobacterium* transformation protocol (Nishimura *et al.,* 2006; Duan *et al.,* 2012). Several scientific reports include transformation of rice cultivars with stress tolerance genes (Todaka *et al.,* 2015; Chen *et al.,* 2017). Some other reports mentioned some attempts to transform Egyptian rice cultivars by using either *Agrobacterium* transformation (Youssef *et al.,* 2011; Hussain *et al.,* 2014; Amer *et al.,* 2016) or by using gene gun transformation (Moghaieb, 2010). Therefore, the current study was concerned to improve drought tolerance of two Egyptian rice cultivars by using *Agrobacterium*–mediated transformation approach. Where, one of *ORPs* gene family (*OPR7*) was used to transform two Egyptian rice cultivars Sakha 106 (Sk106) and Giza 178 (G178).

MATERIALS AND METHODS

Plant materials and grains sterilization

The two Egyptian rice cultivars Sakha 106 (Sk106) and Giza 178 (G178) grains were kindly provided by Rice Research Department, Field Crops Research Institute (FCRI), Agricultural Research Center (ARC), Egypt. G178 was originated from crossing between Giza 175 and Milyang 49. G178 is 62.5% *Indica* and 37.5% *Japonica* cultivars. It is high blast resistant and lodging resistant (Moghaieb *et al.,* 2009). While Sk106 was originated from crossing between Giza 177 and Hexi 30 (Hefena *et al.,* 2016). It is 100% *Japonica*.

The outer coats of mature rice grains of the two cultivars were removed and soaked in 70% ethanol for 10 min. Then they were washed with sterile water. The grains were soaked in 50 % commercial sodium hypochlorite and 0.1% Tween for 30 min with shaking. The grains were then rinsed three times with sterile distilled water (Youssef *et al.,* 2011).

Media

All media compositions were constructed according to Youssef *et al.* (2011). About 25 sterilized grains were partially submerged on a single plate containing solid induction medium (30 g Sucrose, 3.98 g (N6) Basal salts, 100 mg Myo-inositol, 300 mg Casein, 2.8 g L-proline, 1 ml of 1000x N6 vitamins, 2 mg 2,4-D and Gellan Gum 4.0 g, pH 5.7~5. 8). Where the 1000x N6 vitamins composed of 200 mg Glycine, 50 mg Nicotinic acid, 50 mg Pyridoxine HCl and 100 mg Thiamin HCl. The plates were incubated at 28°C in the dark for 4 weeks.

Agrobacterium strain & plasmid construct

The *Agrobacterium tumefaciens* strain LB4404 harboring the pH7-GWF-

OPR7 binary vector (Fig. 1) was used for the transformation process. This strain was kindly provided by Prof. Dr. Peter Nick, Karlsruhe Institute of Technology (KIT), Germany (under the umbrella of cooperation between Egypt and Germany (GERF) projects, funded by STDF). The vector pH7-GWF-*OPR7* is harboring *OPR7*, *eGFP* and *hygromycin* genes (Fig. 1).

***Agrobacterium* culture and co-cultivation**

The *Agrobacterium* transformation was performed according to Nishimura *et al.* (2006). The *Agrobacterium tumefaciens* LB4404 colony containing the pH7-GWF-*OPR7* binary vector was streaked onto a solidified *Agrobacterium* minimal medium (AB medium) (5 g Glucose, 3 g K_2HPO_4, 1.3 g NaH_2PO_4 $2H_2O$, 150 mg NH_4Cl, 10 mg $CaCl_2$ $2H_2O$, 2.5 mg $FeSO_4$ $7H_2O$, 15 g Agar, pH 7.2). After autoclaving, 1.2 ml of sterile 1M $MgSO_4$ $7H_2O$ was added to the media. The media was containing 50 mg Hygromycin, 100 mg Spectinomycin, 300 mg Rifampicillin and 300 mg Streptomycin. The plates were incubated for 2 days at 28°C. The *Agrobacterium* cells were collected with a sterilized spatula and resuspended in co-cultivation liquid medium gently (OD_{600}= 0.2). The rice calli were soaked in the *Agrobacterium* suspension for 15 min. After that, the rice calli were then co-cultured with *Agrobacterium* on solid co-cultivation medium (Induction media and 100 µM Acetosyringone, pH 5.7~5.8) for 3 days at 28°C in the dark in growth chamber.

Selection and regeneration of transgenic rice

The transformed calli were transferred into selective media (Induction media with 50 mg Hygromycin and 300 mg cefotax) while the non-transformed calli were subcultured into induction media with no antibiotics. The *eGFP* transient expression was detected for transformed calli using Fluorescence Microscope (Olympus, Japan). The surviving healthy transformed calli were transferred to regeneration medium (30 g Sucrose, 30 g Sorbitol, 4.4 g MS basal salts, 2.0 g Casein, 1 ml B5 vitamins, 0.02 mg NAA, 2 mg Kinetin, Gellan Gum 4.0 g, pH 5.7~5.8) containing 50 mg Hygromycin and 300 mg cefotax. While the non-transformed calli were transferred into regeneration medium with no antibiotics. The regenerated plantlets were transferred to MS hormone free medium (30 g Sucrose, 4.4 g MS basal salts, 1 ml B5 vitamins Gellan Gum 4.0 g, pH 5.7~5.8) containing either 50 mg Hygromycin and 300 mg cefotax or with no antibiotic for transformed or non transformed calli for rooting formation, respectively. Healthy seedlings were transferred into rice hydroponic containing Yoshida solution (Yoshida *et al.,* 1976) for extensive rooting. After that, the plantlets were planted into plastic pots containing peat moss and vermiculite in equal amounts and grown in a green house for acclimatization and the spikes formation.

Molecular analysis of transformed plantlets

- **DNA isolation and PCR analysis**

DNA was isolated from putative transgenics and non-trangenics according to the manufacturer's protocol of PureLink Genomic DNA Mini kit (K1820-01, Invitrogen, USA). PCR reactions were conducted to amplify *eGFP* (518 bp) as well as Hygromycin resistance gene (*Hyg*) separately according to the EmeraldAmp Max PCR Master Mix kit (Takara, Japan) using gene specific primers (Table 1). PCR reaction conditions: 5 min at 95°C for one cycle; then 40 cycles as followed: 30 sec at 95°C; 30 sec at 52°C for *eGFP* & *Hyg* reactions and followed by 45 sec at 72°C. After the amplification, 10 min at 72°C for final extension then the reactions hold at 4°C. The PCR reactions products were resolved on 1.5% agarose gel.

The primers used in this study are listed in Table (1). The online Primer BLAST tool (NCBI) and Primer 3 software were used for designing the primers and checking its specificity. The primers were synthesized by Eurofins Company, Germany.

- **RNA isolation and cDNA synthesis**

According to the manufacturer's protocol of Trizol solution (Sigma, USA), total RNA isolation from leaf samples of the transformed and non transformed plantlets of both cultivars G178 and SK106 were extracted. The isolated RNA was used in the cDNA synthesis. The cDNA synthesis reactions were carried out using Superscript RT Enzyme (200 units, Invitrogen, Cat No. 18080-044) according to the manufacturer's protocol. The synthesized cDNAs were stored at -20°C for the conventional RT-PCR and Real time PCR (qRT-PCR) reactions.

The conventional PCR reactions were conducted according to the EmeraldAmp Max PCR Master Mix kit (Takara, Japan) manufacturer's protocol. One µl of each Full *OPR7* primer (10 pmol) was used in 25 µl total reaction volume. PCR reaction conditions: 5 min at 95ºC for one cycle; then 40 cycles as followed: 45 sec at 94°C; 30 sec at 60°C annealing temp.; 2 min at 72°C. After the amplification, 10 min at 72°C for final extension then the reactions hold at 4ºC. The PCR reactions were loaded on 1.5% agarose gel.

- **Real Time PCR**

The cDNAs of the transformed and non transformed plantlets of both cultivars G178 and SK106 were used to measure and analyze the expression of *OPR7* gene. The house keeping gene Actin was used to test the transcription reactions and also as Reference gene in real time PCR (RT-PCR) reactions. These reactions were carried out in Mx3005P Real Time PCR system (Stratagen). The RT-PCR reactions were conducted as follows: 5 µl of Quantitech Syber Green master mix (Qiagen); 0.3 µl of each primer *OPR7* (10 pmol) or Actin; cDNA 20 ng; complete the reac-

tions to 10 µl with dH_2O. RT-PCR reaction conditions: 15 min at 95ºC for one cycle (activation step); then 40 cycles as follows: 15 sec at °C; 30 sec at 60ºC; 30 sec at 72°C.

Physiological Analysis

All physiological experiments were carried out on transgenic and non-transgenic plants (control and stressed leaves tissues).

- **Relative water content (RWC)**

Relative water content (RWC) was measured for some transgenic and non-transgenic plants (control and stressed leaves tissues) according to Basnayake *et al.,* (1993). The RWC was calculated using the following formula:

$$\text{RWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Where: FW is the initial fresh weight of leaf tissues, TW is the turgid weight of tissues after 4 hours of incubation in water at room temperature and DW is dry weight after oven drying at °C for 24 hours.

- **Determination of chlorophyll contents**

Chlorophyll (Chl) a, chlorophyll b, total chlorophyll, and carotenoids were determined according to Hassanein *et al.* (2009). Carotenoids, chlorophyll (b) and chlorophyll (a) were measured at 452.5, 644, and 663 nm, respectively. The concentration of each pigment (µg/ml) was estimated using the following equations:

Chlorophyll a (µg/ml) =10.3 × E 663 − 0.98 × E 644

Chlorophyll b (µg/ml) =19.7 × E 644 − 3:87 × E 663

Total chlorophyll = Chlorophyll a + Chlorophyll b

Total carotenoids (µg/ml) = 4.2 E 452.5 − {(0.0264 × Chl a) + (0.426 × Chl b)}. The pigment contents were expressed as µg g-1 dry weight (DW) of leaves

- **Extraction and estimation of proline**

Free proline was determined according to the method described by Bates *et al.,* (1973). The absorbance was measured at 520 nm using spectrophotometer (Spectronic 601, Milton Roy Company). Proline concentration was determined from a standard curve of proline and calculated as µg/g FW of the plant.

RESULTS AND DISCUSSION

Agrobacterium infection, callus induction, regeneration and root formation

Drought is one of the major threats that affect Mediterranean regions. It reduces water availability for agriculture causing harsh damages and negative impacts over economy (Peña-Gallardo *et al.,* 2018). Rice is considered to be a global vital crop; its cultivation requires intensive water therefore rice cultivation is so sensitive to water deficiency and needs an appropriate water management (Silalertruksa

et al., 2017). Plant transformation with drought tolerance related genes like oxo-phytodienoate reductases (*OPRs*) open promises gates to reduce the water need for rice cultivation. It has been proven that *OPRs* could enhance plant tolerance against different abiotic stresses (Li *et al.,* 2011).

Due to its availability at any time of the year and its wide use in rice regeneration (Mostafiz and Wagiran, 2018), mature grains have been used in the current study as efficient explants in rice regeneration through callus culture. As reported previously by El-Shawaf *et al.,* (2011) efficient rice regeneration was obtained by using embryonic calli derived from mature grains of Egyptian cultivars. The Egyptian G178 (75% *indica*) cultivar proved its high regeneration ability and its useful usage as genetic resources for *Agrobacterium* transformation. The embryonic calli have been derived from the mature grains of two Egyptian rice cultivars after incubation on callus induction media. The regeneration step was shown in Fig. (2 A-C). The *Agrobacterium* transformation was preformed according to Nishimura *et al.,* (2006). The Nishimura group established a protocol for *Agrobacterium* transformation that is applicable for *japonica* (difficult to be cultured) and *indica* (easy to be cultured) varieties. The two Egyptian cultivars calli were transformed by pH7WGF-*OPR7* construct (Fig. 1).The acetosyringone concentration was one of the affecting factors in *Agrobacterium* transformation. Therefore, acetosyringone was used in the transformation of the calli from the two rice cultivars according to Youssef *et al*., (2011).

After *Agrbacterium* transformation, the calli were transferred into selection media (Teixeira da Silva and Fukai, 2001). In addition to the hygromycin antibiotic resistance, putative transgenics have been selected using *eGFP* signal assay. After three weeks on the selection media, the signal of the *eGFP* expression was examined using fluorescence microscope to select the transformed calli with green illumination (Fig. 2 G-J). Survived transformed calli transferred into regeneration media (Fig. 2 D-F) then transferred into rooting media after shoots formation (Fig. 2 K). The healthy seedlings were transferred into rice hydroponic containing Yoshida solution (Yoshida *et al.,* 1976) for extensive rooting (Fig. 2 L). After that the plantlets were transferred into plastic pots and grown in a greenhouse for acclimatization and the spikes formation (Fig. 2 M). The number of putatively transformed explants, the number of regenerated transgenic plantlets, the number of the obtained transgenic rice lines and the number of RT-PCR positive plantlets are presented in Table (2).

As mentioned before, using *OPR's* in plant transformation was performed once successfully to enhance the tolerance of tomato plants against salt stress (El Nagar, 2013). To ensure the *OPR*7 gene transformation and its efficiency, our putative transgenic plants subjected to sever-

al molecular and physiological investigations.

Molecular analysis for the transgenic rice plants

- **Conventional PCR and Real time PCR analysis**

To confirm the presence of the T-DNA containing the *ORP7*, PCR reactions were carried out on the putative transformed plantlets. Two primers combinations were designed and used for the detection of *hyg* and *eGFP* genes. The resulted amplicons (474 bp) for the *hyg* gene and (518 bp) for the *eGFP* gene were shown in Figs (3 and 4), respectively. Moreover, RT-PCR reactions were done with putative transgenics using *OPR7* primers revealed the expected cDNA band size (1182 bp) for full length *ORP7* gene (Fig. 5).

Conventional RT-PCR reactions using actin and *OPR7* real time primers were carried out to test them before conducting the real time reactions. As expected in Table (1), the PCR revealed two products; 150 bp with actin primers and 156 bp with *ORP7* primers Fig. (6 A and B). The differential expression of *OPR7* gene in transgenic plants was represented as number of fold changes compared with the relative non transgenic plants. Relative gene expression ratios (RQ) between transgenic and non-transgenic plants were calculated according to this formula RQ = $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). RT-PCR of transgenic rice events of two cultivars (SK106 and G178) revealed that *OPR7* was expressed under drought and normal conditions compared with the non-transgenic ones. The expression of *OPR7* was higher in drought treated transgenic lines than the non-transgenic plants (Fig. 7) indicating that these transgenic events are not only successfully transformed but also have the *OPR7* gene with high expression rates.

Physiological analysis for transgenic rice

The response of rice transgenic events and non-transgenic control cultivars was evaluated by relative water content, photosynthetic pigments after exposure to drought stress.

- **Relative water content**

Relative water content of the transgenic plants as well as non-transgenic were investigated under drought stress and normal conditions. The transgenic plants had the lowest decrease in relative water content in compare with non-transgenic plants (Fig. 8 A), which indicate the enhancement of drought tolerance in the transgenic plants which agrees with Keyvan (2010) conclusion from his experiments on drought stress where he found that the cultivar with the highest drought tolerance and yield stability had the lowest decrease in relative water under drought stress.

- **Photosynthetic pigments**

The photosynthetic pigments of the transgenic plants were investigated under

drought and normal conditions. Carotenoids, Chlorophyll a and chlorophyll b concentration were remarkably increased under drought condition in transgenic plants Fig. (8 B-D). On the other hand, Photosynthetic pigments of transgenic plants had significantly higher levels as compared to non-transgenic plants. This increase in the photosynthetic pigments enhances light harvesting in order to protect plant from oxidative damage and abiotic stresses (Jaleel, 2009).

• Proline content

Accumulation of Proline enhances the plant drought tolerance (Dar *et al.,* 2016), therefore Proline (μM/g FW) content were investigated under drought stress for transgenic and non-transgenic (G 178 and SK 106) cultivars Fig. (8 E). The content of Proline increased in both transgenic and non-transgenic plants in the two cultivars under drought treatment. The proline content for drought treated transgenic SK106 plant was 2.7 fold where it was 3.36 fold for drought treated transgenic G178 plant.

In agreement with the El Nagar (2013) results, the molecular analysis has revealed the expression of *OPR7* in both Egyptian cultivars (SK106 and G178). Moreover, the increase in relative water content, carotenoids, chlorophyll content, proline content in the transgenic rice Egyptian cultivars demonstrate that the *OPR7* enhances the drought tolerance in Egyptian cultivars (SK106 and G178).

• Segregation analysis of T1 progenies

The T_1 grains from each transgenic rice line were sterilized and germinated on jars with MS selection media (Fig. 9). The transgenic grains were successively germinated while the non-transgenic ones couldn't germinate. The survived plantlets were transferred into hydroponics containing Yoshida solution (Yoshida *et al.,* 1976) for extensive rooting. Then the plantlets were transferred into pots in green house.

To check the transgene stability in T1 plants, PCR reactions were carried out on DNA extracted from the leaves of the T1 plants of both transgenic lines. They were screened using the *hyg* primers in PCR reactions. The *hyg* PCR fragment is 474 bp in length Fig. (10). To prove *OPR7* gene expression in T_1 plants of the transgenic rice lines from both cultivars, cDNAs were prepared from some transgenic lines T_1 plants after RNA isolation. The PCR reactions were loaded on 1.5% agarose gel. PCR amplicon was 1182 bp as expected size for the full length *ORP7* . gene Fig (11).

The *OPRs* genes are essential for both biotic and abiotic stress responses (Al-Momany and Abu-Romman, 2016). The present study was focused on the role of *OPR7* in drought stress. The results were in agreement with Agrawal *et al*. (2003) and Tani *et al.,* (2008) which showed that the *OPR1* and *OPR7* were up regulated in rice under drought stress. Moreover, the increased *OPR1* expression

under salinity on transgenic tomato plants was proved by El Nagar, (2013).

In conclusion, *OPR7* gene was successfully introduced into Egyptian rice cultivars G 178 and SK 106. This was confirmed by means of PCR; RT-PCR; green fluorescence images and hygromycin selection for T_0 and T_1. Moreover, measuring the chlorophyll a; chlorophyll b; total chlorophyll; carotenoids and proline content proved the change on their contents related to drought treatment.

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SUMMARY

Drought represents one of the major challenges that face agriculture in Egypt especially rice. Therefore, development of drought tolerant rice cultivars is an essential step to overcome the drought stress and yield loss. Embryonic calli derived from mature grains of two Egyptian rice cultivars G178 and Sk106 have been transformed by Oxo-phytodienoate reductase 7 (*OPR7*) gene using *Agrobacterium*–mediated transformation system. The results of transgenic plantlets analysis showed that *OPR7* gene was successfully integrated into some transgenic T0 and T1 plantlets of both rice cultivars. Moreover, *OPR7* gene expression in genetically modified plants was confirmed by real time PCR. The examination of the photosynthetic pigmentation, proline content and relative water content revealed higher levels in the drought treated transgenic plants than the non transgenic plants.

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Table (1): List of used primers.

	Name	Primer sequence	Expected
1	eGFP-F	5' ATG TGA TCG CGC TTC TCG TT 3'	518 bp
2	Hyg-F	5' ATG CCG GTC GAT CTA GTA ACA 3'	474 bp
3	For-OPR7-F	5' ATGGATCGGCCGCCGCCGGAT 3'	1182 bp
4	For Real		
5	Act Rice-F	5’ CAG TTC TGA CCA ATC AAA CGA	150 bp

Table (2): The numbers of the putatively transgenic explants; the induced calli; the regenerated and selected transgenic plantlets; the obtained transgenic rice lines and RT-PCR positive plantlets.

Type of explant	No. of Callus	Percentage (%)
Putative transgenics	2570	
Induced calli	1978	76.9 %
Regenerated and selected transgenic plantlets	772	30.0
Transgenic lines (putative events)	103	13.3
+ve RT PCR (transgenic events)	22	2.8

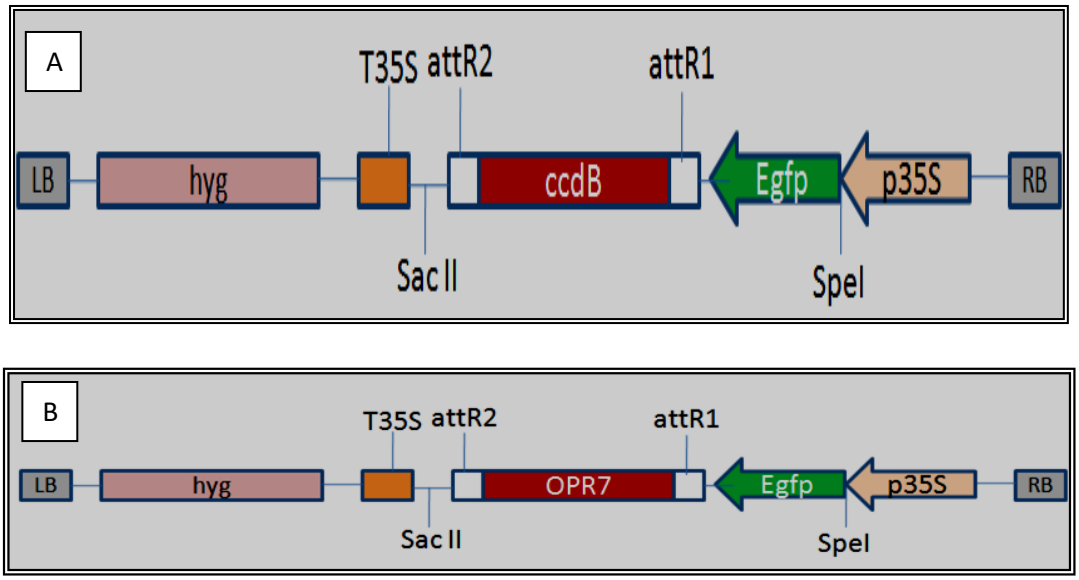

Fig. (1): Schematic representation of pH7WGF2.0 (A) and pHWGF2.0-*OPR7* (B) binary vector cassette.

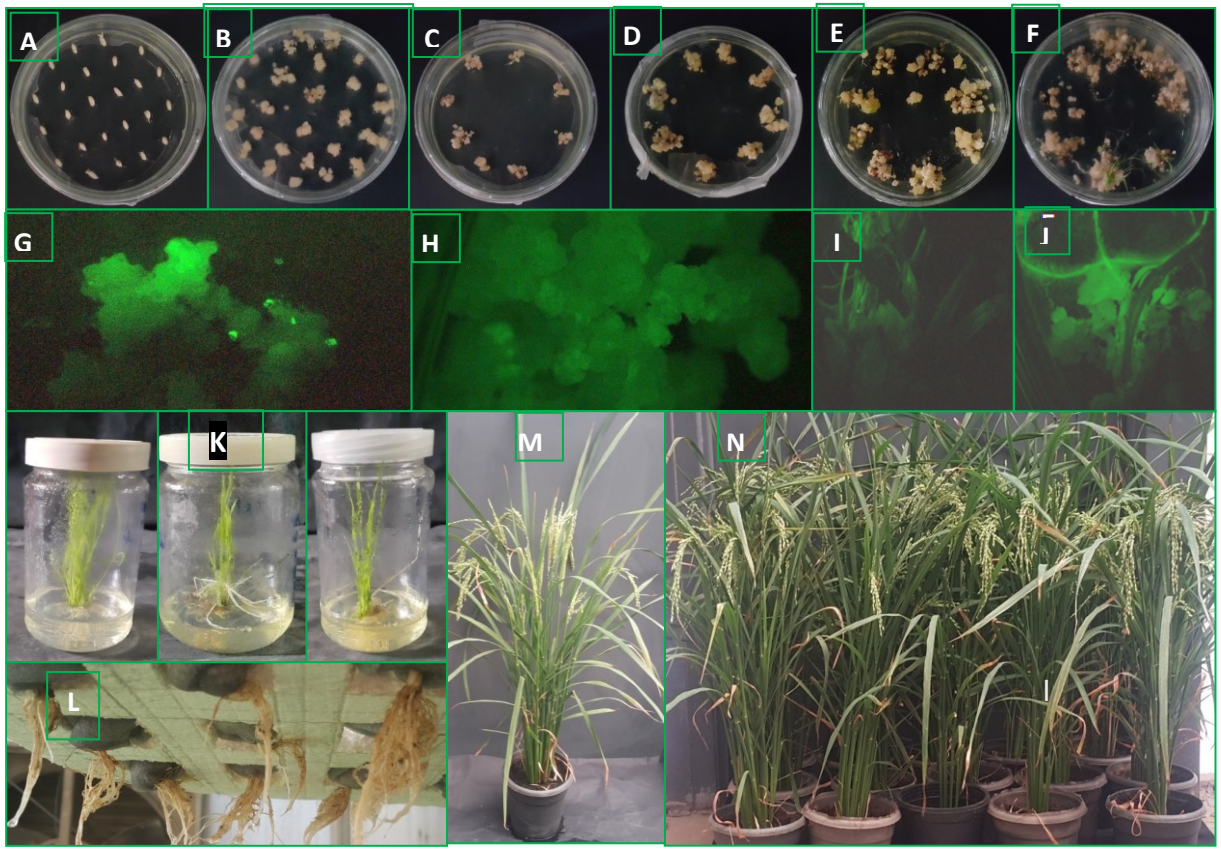

Fig. (2): Production of transgenic Egyptian rice. A - C: mature rice grains and calli induction; D - F: calli on regeneration media; G - J: eGFP assay of calli; K: regenerated transgenic plantlets on jars; L: intensive rooting production on Yoshida solution and M - N plants of transgenic rice in soil.

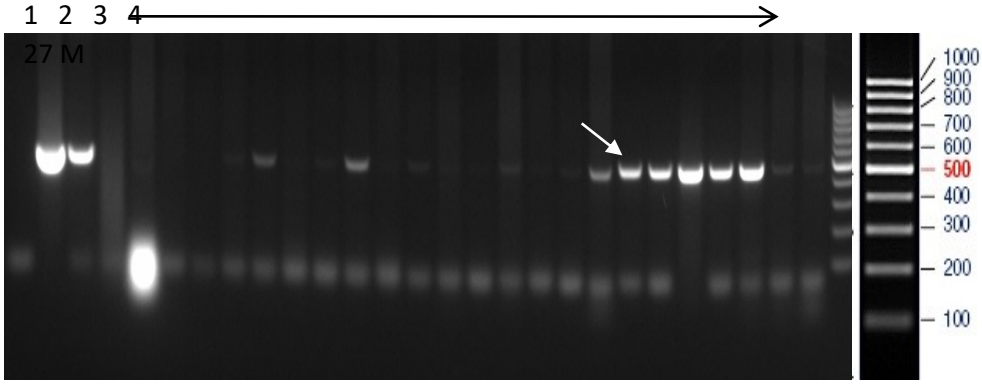

Fig. (3): PCR analysis of Hygro insertion in transgenic rice plantlets using specific hygromycin primers. Lane 1: –ve Control; lane 2: pH7GWF-*OPR7* as +ve control; lane 3: pGEM-T-*OPR7* vector as +ve control; lanes 4-27 some transgenic rice plantlets and lane 28: 100 bp marker (Fermentas).

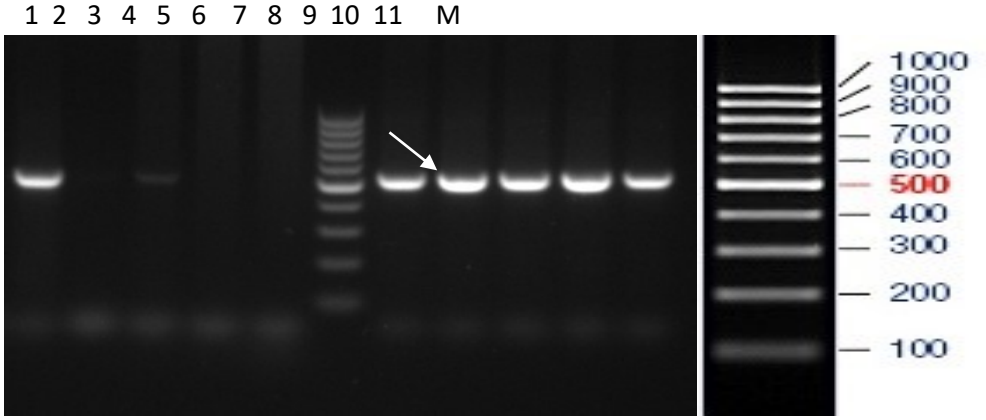

Fig. (4): PCR analysis of *eGFP* insertion in transgenic rice plantlets using specific *eGFP* primers. Lane1: pH7GWF-*OPR7* vector as +ve control; lane 2: –ve control; lanes 3-5: some transgenic rice plantlets; lane 6: 100 bp marker (Fermentas) and lanes 7-11: some transgenic rice plantlets

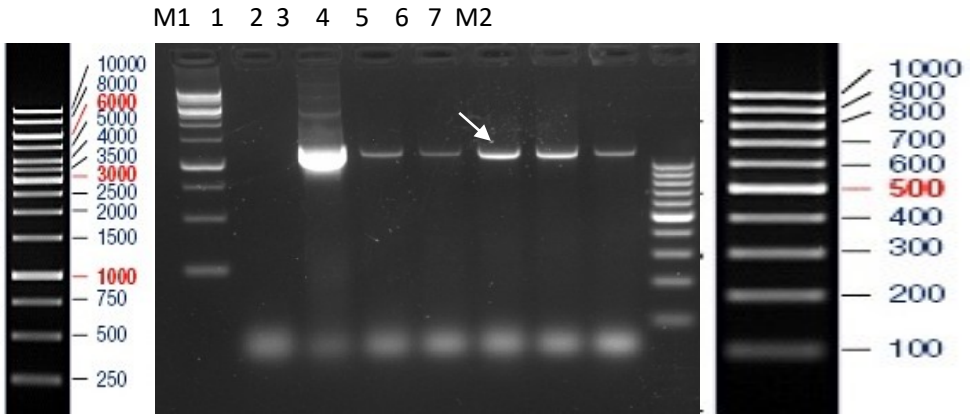

Fig. (5): PCR analysis of *OPR7* insertion in transgenic rice plantlets using specific *OPR7* primers. M1: 1kb marker (Fermentas); lane 1: –ve control; lane 2: pH7WGF-*OPR7* vector; lanes 3-7: some transgenic rice plantlets. M2 is 100 bp marker (Fermentas)

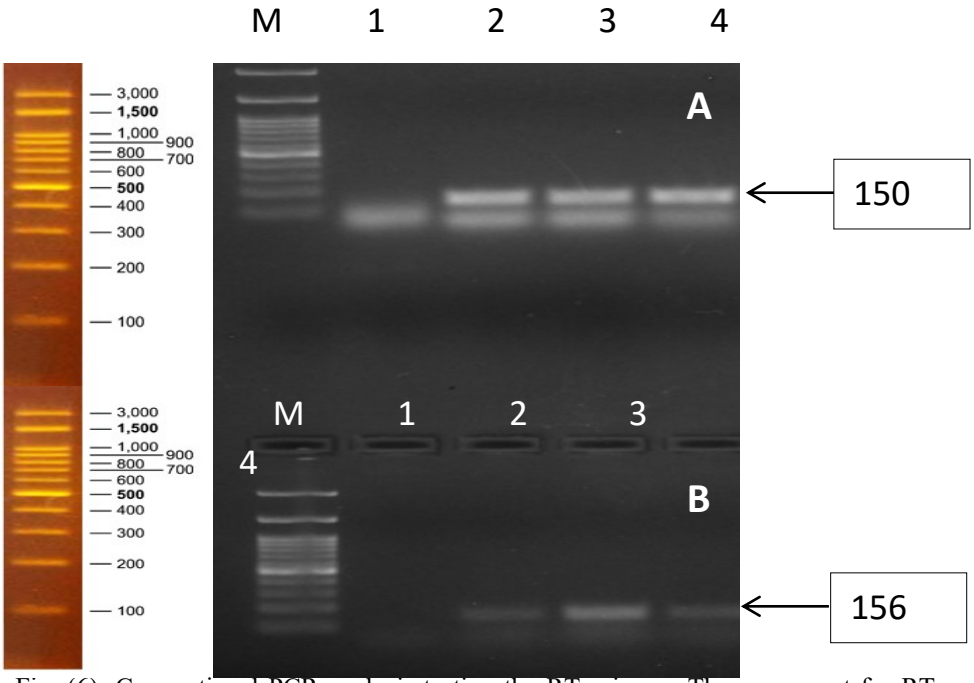

Fig. (6): Conventional PCR analysis testing the RT primers. The upper part for RT-*actin* primer and lower part for RT-*OPR7* primers. Marker is 100 bp ladder (GeneDirex). Lane 1:-ve Control; lanes 2-4 some transformed plants.

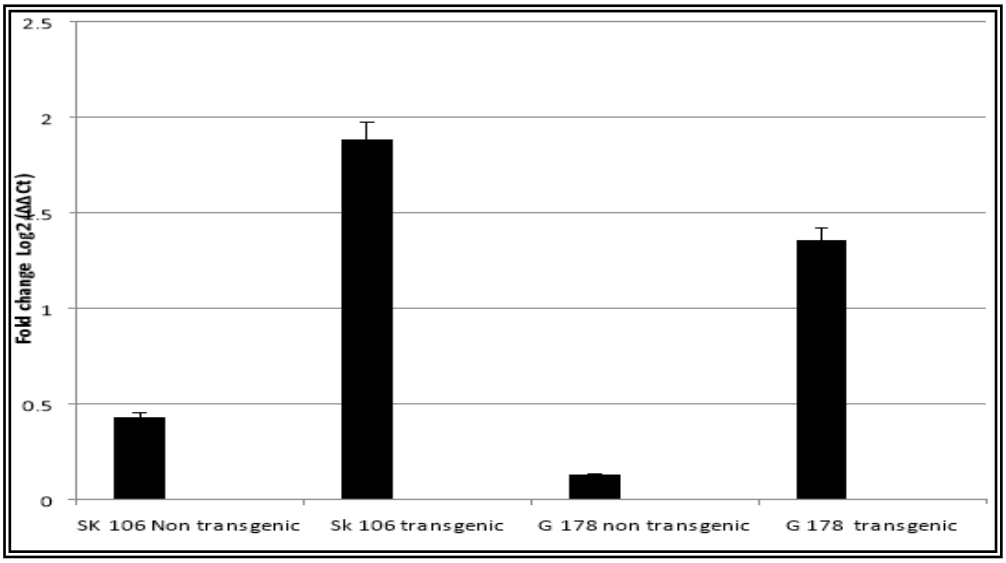

Fig. (7): Analysis of *OPR7* expression in transgenic rice cultivars lines by real time PCR, where *actin* is used as reference gene.

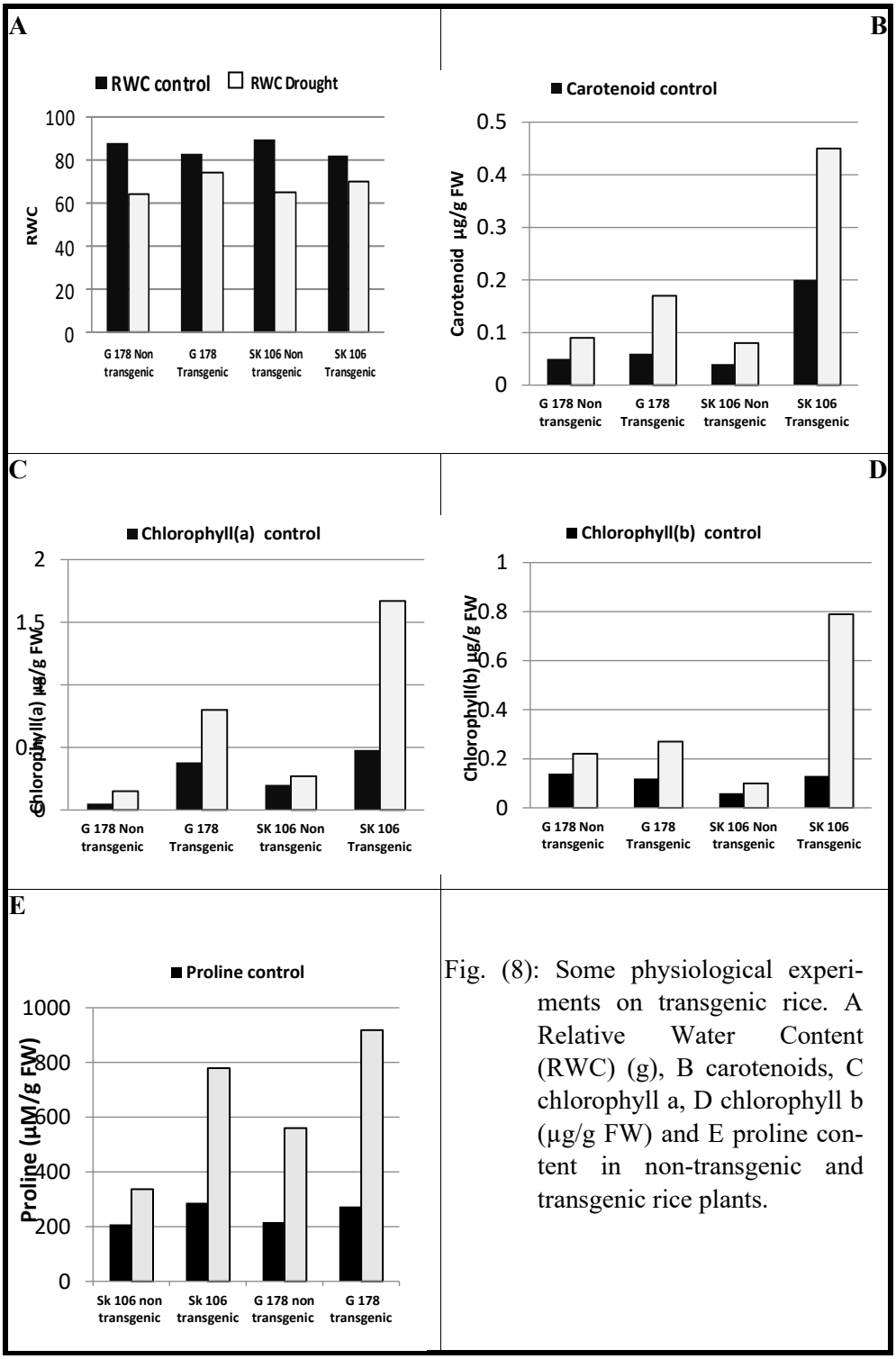

Fig. (8): Some physiological experiments on transgenic rice. A Relative Water Content (RWC) (g), B carotenoids, C chlorophyll a, D chlorophyll b (µg/g FW) and E proline content in non-transgenic and transgenic rice plants.

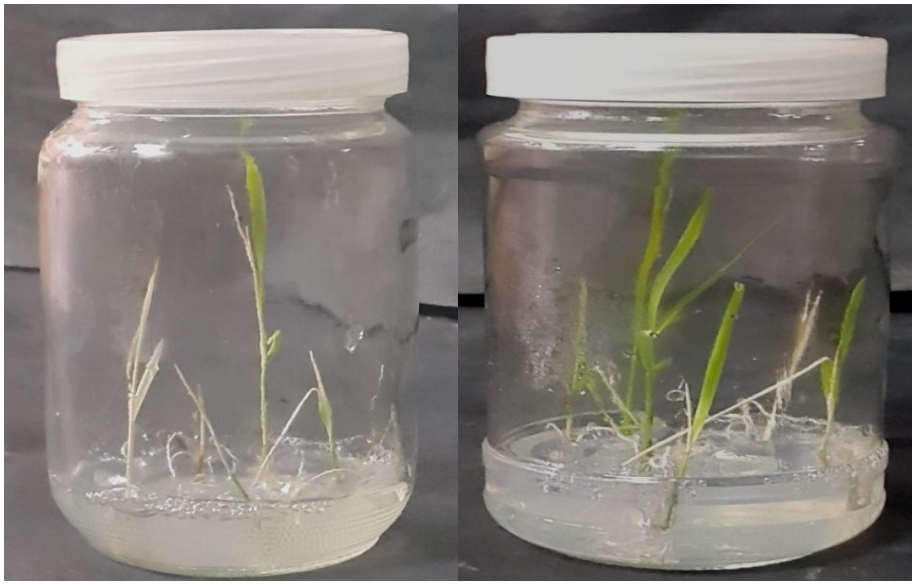

Fig. (9): Selection of transgenic grains by germination on MS media containing Hygromycin.

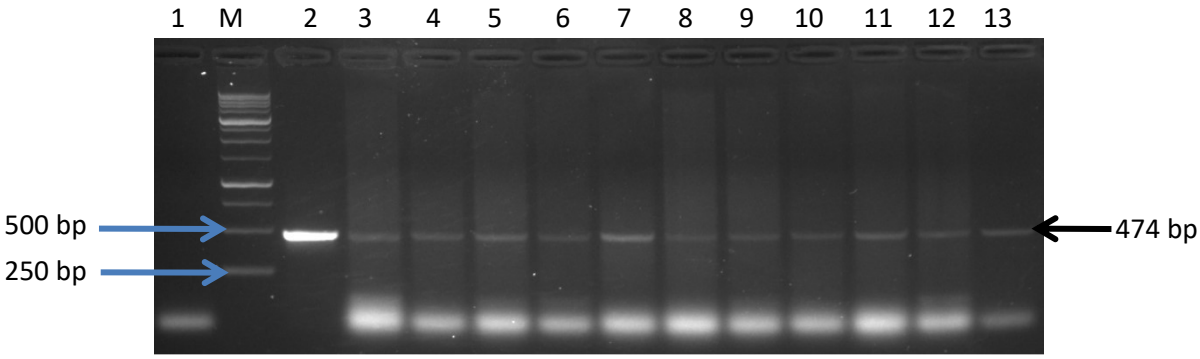

Fig. (10): Screening of some transgenic rice lines T1 by PCR using hygromycin primer. Where M is 1kb marker (Fermentas); lane 1 represents –ve control; Lane 2 represents +ve control; lanes 3 –13 represent the transgenic rice lines. Hygromycin band was 474 bp.

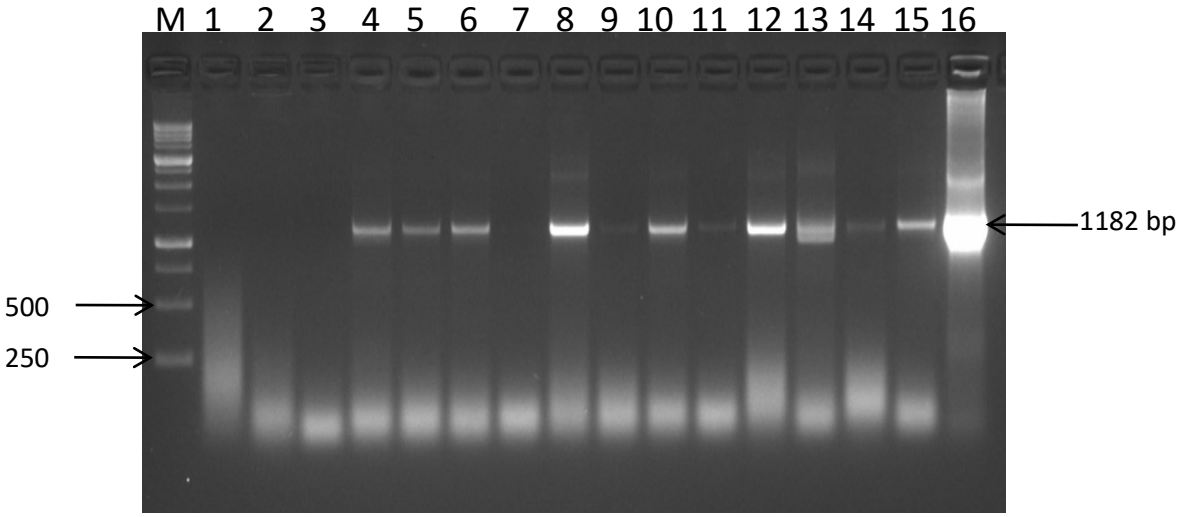

Fig. (11): PCR analysis of *OPR7* insertion in transgenic rice lines T1 using specific *OPR7* full primers. M1: 1kb marker (Fermentas); lane 1: –ve control; lanes 2-15: some transgenic rice lines T1; lane 16: pH7WGF-*OPR*7 vector as +ve control.