GENETIC TRANSFORMATION IN RICE

By

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ABSTRACT

This study was conducted to develop a regeneration and transformation protocol in an attempt to improve heat tolerance of Egyptian rice. The procedure permitted the regeneration of transgenic rice plants in only two months. High percentage of callus induction was obtained when mature seeds were cultured on modified N_6 medium under light condition. 2mg/l 2,4-D was found to be the most beneficial concentration for callus induction of the four tested Egyptian rice cultivars. Shoot regeneration was achieved by transferring the embryogenic calli into modified MS medium. Two mg/l kinetin were chosen as the best concentration for shoot regeneration. Since G178 cultivar showed the highest regeneration capacity among the other tested cultivars, it was selected for transformation experiments. Two to three week-old scutellum calli served as an excellent starting material for transformation. These explants were co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring Athsp101 gene driven by the maize ubiquitin promoter and hygromycin resistance gene driven by CaMV35S promoter. factors affecting *Agrobacterium*-mediated transformation of embryogenic callus were optimized. Inclusion of acetosyringone proved to be indispensable for successful transformation. Optimum co-cultivation period was 3 days. Pre-culture of calli in fresh medium for four days prior to infection with *Agrobacterium* was important to obtain efficient transformation rate. Polymerase chain reaction and Southern blot analysis confirmed the presence of the transgenes. A comparison between transgenic plants and non-transgenic control plants after exposure to different levels of high temperature stress was reported. The transgenic rice showed significantly better growth performance in the recovery phase following the stress. The protein and isozymes banding profiles of the transgenics and non-transgenic control plants after exposure to 28°C and 45°C for different periods (1h, 3h and 6h) were determined.

Key words. Rice- Regeneration- *Agrobacterium*- transformation Thermotolerance

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INTRODUCTION

Rice (*Oryza sativa* L.) is arguably the world′s most important food crop. Over half of world′s populations depend on rice as their stable food (Li-na *et al*., 2010). In Egypt, rice is considered the most popular and important field crop for several reasons: as a stable food after wheat for the Egyptian population, as a second exporting crop after cotton, as a land reclamation crop for improving the productivity of the saline soils widely spread in North delta and coastal area, and finally it is a social crop in which all farmers family member could gain money during its growing season (since rice is cultivated on about 25% of total cultivated area in the summer season) (Aidy *et al*., 2006).

The earth's climate is predicted to change due to the build-up of greenhouse gases, primarily Co_2, methane and nitrous oxide. Global mean surface temperatures have increased in the late 19^{th} century. The 20^{th} century's ten warmest years have occurred in the last 15 years of the century (http://www.epa.gov). It is estimated that global temperature will rise 0.3°C per decade (Jones *et al*., 1999) reaching to approximately 1.4-5.8°C above the present value by year 2100 and leading to global warming. Rising temperatures may lead to altered geographical distribution and growing seasons of agricultural crops by allowing the threshold temperature for the start of the season and crop maturity to reach earlier (Porter, 2005). Global warming would significantly affect agricultural ecosystems.

High temperature stress is considered as one of the major stresses on crop plants (Grover *et al*., 2000). Rice is sensitive to high

temperature stress at almost all the stages of its growth and development. The optimum temperature for rice growth is 25-31°C depending on the variety. According to estimates, yield of rice declines by 10% for every 1°C increasing in growing period minimum temperature in the dry season (Peng *et al*., 2004). It is therefore evident that the overall growth, production and quality of rice are severely affected by high temperature stress.

The response of plants to heat stress results in changes in the level of enzymes, cellular membrane structure, photosynthesis activity and protein metabolism (Singla *et al*., 1997). At the molecular level the synthesis of heat shock proteins (hsps) represents the most interesting, but still not completely explained, aspect of heat shock response. These proteins play the essential role of preventing or minimizing the deleterious effects of heat at the cellular and molecular levels. Moreover, they help cells in recovering from the stress during the post stress phase (Larkindale *et al*., 2005). Most of these proteins act as molecular chaperones by preventing aggregation as well as promoting reactivation of heat-damaged proteins (Hu *et al*., 2009;Tripp *et al*.,2009;Gupta *et al*., 2010).

Hsp100 belongs to a unique class of molecular chaperons that function to resolubilize protein aggregates formed as an effect of the heat stress (Agarwal *et al*., 2003; Lee *et al*., 2004, 2005). Hsp100 proteins, in particular, have emerged as strong candidates for enhancing high temperature tolerance in plants in the recent years .Hong and vierling (2000) identified four separate genetic loci, hot 1-4, involved in acquired thermotolerance. The hot 1 mutant (with a

mutation in the Athsp101 gene) was found to be defective in tolerance to high temperature. Additional evidence has been provided by the manipulation of the expression from *Athsp101* in *Arabidopsis*. The inhibition of Athsp101 expression by antisense constructs or through cosuppression led to the impairment in the ability of plants to withstand high temperature stress (Queitsch *et al*., 2000) .On the other hand, overexpression of this gene resulted in a high-level of basal thermotoleyance in transgenic plants (Queitseh *et al*., 2000). Clear evidence of the protective role of *Athsp101* and its involvement with thermotolerance was shown in rice in which the survival to high temperatures and induction of thermotolerance were strictly bound to the presence of *Athsp101* (Katiyar-Agarwal *et al*., 2003).Recently, Myouga *et al*., (2006) reported that an *Arabidopsis* chloroplast-targeted Hsp101 homologue has an essential role in chloroplast development as well as heat-stress response. Nevertheless, the essential functions which are protected or repaired by *Hsp101* remain to be elucidated.

Genetic transformation of rice enables breeders to rapidly design new varieties by the introduction of desired alien genes into existing commercial lines. Desirable genes from any species in *planta* irrespective of their evolutionary and taxonomic status and other organisms such as bacteria, virues, animals and even humans can be transferred to the rice genome. Different transformation methods have been used successfully to transfer DNA to rice such as DNA uptake *via* PEG (Datta *et al*., 1990), electroporation (Biswas *et al*., 1994), *Agrobacterium*-mediated transformation (Ozawa and Takaiwa 2010) and particle bombardment (Saker *et al*., 2006). However, genetic

transformation of rice through the use of *Agrobacterium* is a favored approach as it enables the transfer of DNA with defined ends, minimal rearrangement, higher expression of target genes, integration of a small number of copies of the gene, minimal sterility of generation, and more importantly, the possibility that even large segments of DNA can be efficiently transferred (Hellens *et al*., 2000). Although rice is not the natural host of *Agrobacterium*, *Agrobacterium*-mediated transformation has now become an efficient and commonly used method in rice transformation since rice transformation succeeded in different varieties as well as several laboratories after 1994 (Hiei *et al*., 1994; Rashid *et al*., 1996).

In view of increasing incidences of temperature fluctuations and constant threat of the global warming, production of high temperature tolerant transgenic rice is of utmost importance. The present study focused on developing an efficient and reliable method for regeneration of some Egyptian rice cultivars and optimizing various parameters for the maximum gene transfer rates of Egyptian rice cultivar Giza178 using *Agrobacterium*-mediated transformation. In addition, the Athsp101 gene was used for transforming rice explants in an attempt to produce transgenic rice plants tolerant to high temperature stress.

REVIEW OF LITERATURE

1. Plant regeneration in rice

Many features of biotechnology today are directed towards the improvement of conventional plant breeding processes, such as introduction of novel genes by genetic transformation, protoplast fusion for the production of male sterile lines, haploid generation for attaining rapid homozygosity and somaclonal variation for introducing increasing trait variability (Hoque *et al*., 2007; Ramesh *et al*., 2009). Efficient plant regeneration *in vitro* is essential for the successful utilization of biotechnology in rice crop improvement (Dabul *et al*., 2009). Genetic transformation is strongly dependent on the genotype and the availability of efficient plant regeneration abilities; Indica cultivars are recalcitrant to various biotechnology advances. Identification and screening of useful cultivars for embryogenic callus formation and subsequent plant regeneration *in vitro* are key steps in rice genetic improvement program through application of biotechnology (Hoque and Mansfield, 2004).

Among many factors which influence callus induction and plant regeneration, genotype and nutrient media composition have been the most important ones. Numerous reports have been published regarding rice tissue culture, but there is a lot of genotype dependence and regeneration in indica rice is still a difficult task. (Kumaria *et al*., 2001). In rice, *in vitro* plant regeneration has been obtained from almost all types of explants (Jain *et al*., 1996). However, significant

variation was observed in embryogenic callus production, somatic embryogenesis and subsequent plant regeneration from different origins. The use of mature seed embryos as starting material for *in vitro* regeneration has distinct advantages over other explants such as supply of plant material without restriction of season and geographical environment, and with easy operation and less infection by microorganisms (Li-na *et al*., 2010). Moreover, embryogenic calli obtained from mature seed embryos are efficient in rice transformation (Kant *et al*., 2007; Kumar *et al*., 2010).

Jose' Pons *et al*. (2000) tested the addition of amino acids and the effect of two macronutrient solutions MSD and N_6D to the basal callus induction medium in three Spanish rice varieties, Senia, Tebre and Bahia. The results indicated that the amino acids enhanced the production of embryogenic callus in Tebre and Senia whereas in the case of Bahia, embryogenic callus, which gave rice to a high rate of differentiated shoots, was induced without amino acids. The macronutrient solution had also to be adjusted for each variety. Pre-regeneration treatment with ABA significantly improved the media in which the embryogenic callus was induced. In a comparison of growth regulators, BA yielded more shoots than kinetin in all varieties whereas the effect of the auxins NAA or IAA was dependent on the variety.

Meneses *et al*. (2005) performed two independent experiments in order to increase the embryogenesis regeneration process obtained from somatic embryos of the indica rice variety CR-5272 (*Oryza sativa* L.). Two independent experiments were performed. The first

experiment studied the effect of combination of three concentrations of the gelling agent Phytagel (1.8, 2.4, and 3 g/l) and four 2,4-D concentrations (2.26, 4.52, 6.78, and 9.05 μM) on the induction and subsequent regeneration of embryogenic calli. On the second experiment, the pre-regeneration phase was modified; calli were subjected to darkness or diffuse light conditions for one, two, and three weeks. In embryogenesis induction, 35% calligenesis was obtained using the MS culture medium supplemented with 6.78 μM of 2,4-D and 2.4 g/l Phytagel , whereas on the control (MS medium supplemented with 9.05 μM of 2,4-D and 3 g/l Phytagel) 24% calligenesis was obtained. In addition, regeneration percentages were improved (22% and 16% for calli induced with the above treatments, respectively). Furthermore, in light exposure experiments, the best result was obtained by exposing the embryogenic calli to darkness for one week in pre-regeneration, followed by direct light exposure during the regeneration phase.

Ge *et al*. (2006) established two media for callus induction and subculture, respectively, in tissue culture of *indica* rice by manipulating plant growth regulators, organic components and salts within the culture media. The modified media could guarantee the production and proliferation of a great number of embryogenic calli with high regeneration capacity from mature seeds representing different indica rice germplasms. The calli obtained from this system should be ideal material for *Agrobacterium* mediated transformation. The results suggested that this optimized tissue culture system will be widely applicable for the tissue culture of indica varieties.

Saker *et al*. (2006) reported an improved regeneration system for some superior Egyptian rice genotypes. The developed system involves the proliferation of highly regenerative embryogenic callus from either mature or immature embryo explants onto MS medium containing 3 mg/L 2, 4-D (callus induction medium, CIM). Differentiation of embryogenic callus into plantlets occurred onto MS medium supplemented with 2 mg/l Kinetin + 1mg/l NAA (shoot recovery medium, SRM). The regeneratants were rooted onto basal MS medium and easily acclimatized to *ex vitro* conditions. Although all investigated genotypes responded to this regeneration system, the regenerative capacity of some genotypes (Giza 177 and Giza 178) showed the highest regenerative capacity, while others (Giza 171, Giza 172 Giza and 175) showed the lowest response. .

Yookongkaew *et al*. (2007) established a system of multiple shoot regeneration from rice shoot apical meristem. By the use of MS medium containing 4 mg/l thidiazuron (TDZ), multiple shoots were successfully developed directly from the meristem without an intervening callus stage. All rice cultivars tested responded well on the medium and regenerated to plantlets that were readily transferred to soil within 5–8 weeks.

Dabul *et al*. (2009) screened a broad range of rice germplasm (thirty- three entries) for *in vitro* rapid regeneration. Entries that exhibited between 50% and 90% regeneration frequencies include 'Taipei-309' 'Super Dwarf ' 'Norin ' (japonica types), PI 312777, 'Ali Combo ' (*indica* types), 'STG-S' and 'LA3' (red rice types). One third of the entries tested were at least two times better at regeneration than

the often cited regenerator 'Nipponbare.' Those entries showing at least 85% frequency of greening or somatic embryo formation at 15 or 30 d on regeneration medium ultimately produced whole plants after 45 d on regeneration medium at high frequency (at least 40%); those entries not reaching the 85% threshold of greening by Days 15 or 30 exhibited moderate (15-40%) to low (less than 10%) frequency of whole plant regeneration. This greening response suggests the means for an early prediction system for identification of useful rice regenerator lines, which would be beneficial for high-throughput screening of germplasm as well as for decreasing the time and cost of *in vitro* culture.

Ghareeb *et al*. (2009) reported a high frequency plant regeneration from mature seed-derived calli of rice. An experiment with five Egyptian rice cultivars (Giza 159, Giza 171, Giza 172, Giza 176 and Reiho) showed that callus induction and growth were significantly affected by genotype and medium composition. Murashige and Skoog(1962) (MS) medium supplemented with 2 mg/l of 2, 4-dicholorophenoxy acetic acid (2, 4-D) gave the highest incidence of callus induction for 'Giza 176' and Reiho, respectively. The regeneration frequencies were dependent on genotype, 6-benzylaminopurine (BAP) concentration, and callus type. 'Reiho' and 'Giza 176' showed the highest callogenesis ability(100%), when calli from 'Giza 176' and Reiho' were induced on 2 and 1.5 mg/l 2,4-D, respectively, and subsequently regenerated on medium supplemented with 0.5 mg/l of 1-naphtaleneacetic acid (NAA) and 3 mg/l BAP. Thus, it is misleading to consider the growth dynamic of callus as the