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Assessment of various factors for high efficiency transformation of Egyptian rice involving DREP2A gene

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Abstract: This study was conducted to provide a well-adopted technique for regeneration and transformation of Egyptian rice cultivars as a first step in improving their heat tolerance. Among different sugar types, maltose (at 4% concentration) was found to provide the highest percentage of callus induction. 9g/l agar was found to be the most beneficial concentration for solidifying the medium. Two mg/l kinetin plus 0.2mg/l NAA were chosen as the best concentration for shoot regeneration. Since Sakha104 cultivar showed the highest regeneration capacity among the two tested cultivars, it was selected for transformation experiments. Two to three week-old scutellumcalli were co-cultivated with *Agrobacterium tumefaciens*harboring DREB2A gene driven by the CAMV35S promoter, hygromycin resistance gene and GUS gene. OD600=0.8 proved to be the best concentration of *Agrobacterium* for Egyptian rice. Optimum co-cultivation period was 3 days. 25 °C was found to be the best temperature for co-cultivation. Polymerase chain reaction and GUS assay confirmed the presence of the transgenes.
Key words:*Oryza sativa* L. ,Regeneration, *Agrobacterium*, Transformation, DREP2A.

Introduction

Rice (*Oryzasativa* L.) is one of the most important food crops in the world, providing a stable diet for over half of the world's population[1]. It is the second most widely cultivated cereal in the world, after wheat. In Egypt, rice crop is even more important and plays a significant role in Egypt strategy for sustaining the food self-sufficiency. Moreover, it is an important source for hard currency since it is the second exporting crop after cotton[2].

Climate change is one of the greatest challenges in today's world. It is estimated that global temperature will rise 0.3°C per decade[3] reaching to approximately 6ºC above the present value by year 2100 and leading to global warming. High temperature stress, which expected to be more severe, is considered as one of the major stresses on crop plants[4]. Rice is sensitive to high temperature stress at almost all the stages of its growth and development. Rice grain yields decline by 10% for each 1ºC increase in minimum temperature during the growing season[5].

Previously, the dehydration-responsive element binding protein (DREB)/C-repeat binding factor (CBF) family were considered the classical transcriptional regulators involved in plant responses to drought, salt and cold stress. Recently, a novel pathway was demonstrated in which DREB2A gene regulates the heat stress response and contributes to the development of thermotolerance[6].

Biotechnological tools including genetic transformation enable breeders to rapidly design new varieties by the introduction of desired alien genes into existing commercial lines. However, the main problem about applying genetic transformation in rice is finding an effective tissue culture system[7]. Among many factors, culture medium and genotype are key steps in rice genetic improvement through application of biotechnology[8].

Although many optimized tissue culture media for rice were published in the literature, they are largely genotype-dependent[9]. So far, no universal medium adaptable to all rice genotypes has been developed. In addition, the difference in the competence of *Agrobacterium* to infect a specific tissue or genotype is still a major drawback in utilizing *Agrobacterium* routinely for the introduction of the gene(s) of interest in rice. For these reasons, optimization of specific types and components of media for specific rice cultivars are required before applying genetic transformation methods[10, 11]. Keeping in view the above facts, the main aim of this study was to develop a regeneration and transformation protocol for Egyptian rice in an attempt to improve their heat tolerance by overexpression of DREB2A gene.

Experimental

Plant material and surface sterilization of seeds

Seeds of Egyptian rice cultivars Sakha104 and Giza178 were kindly provided by the Rice Research Program; Field Crop Research Institute; Agricultural Research Center; Ministry of Agriculture; Egypt. They were first sterilized with 70% ethanol for 2 minutes and then with 50% commercial colrox (5% NaOCl) for 30 minutes. The seeds were further washed three times with sterilized distilled water.

Influence of different sugar types on callus induction

This experiment was carried out to determine the type and level of carbon source that most beneficial for callus induction. Mature seeds of the two Egyptian rice cultivars were cultured on callus induction medium (CIM) (Table 1) supplemented with different types and concentrations of sugars. Maltose, glucose and sucrose with 3%, 4% and 5% were tested for their efficiency. The results were taken after three weeks. The responded explants that succeeded to induce callus were scored and also the explants that did not show any response or failed to keep alive were also scored.

Table 1. Media used for tissue culture and transformation

Medium (Type)	**Composition**
Callus induction medium (CIM)	N_6 salts and vitamins [47], 40 g/l maltose, 0.3 g/l casamino acids, 2.8 g/l proline, 2mg/l 2.4-D, 7 g/l agar, pH 5.7.
Co-cultivation medium (CCM)	CIM + 100 μ M acetosyringone
Selective medium (SM)	CIM + 50 mg/l hygromycin + 500 mg/l cefotaxim.
LB medium	[48]
Shoot regeneration medium (SRM)	MS salts and vitamins [49], 30 g/l sucrose, 30 g/l sorbitol, 2 g/l casamino acids, 2 mg/l kinetin, 0.2 mg/l NAA, 7 g/l agar, pH 5.7.
Rooting medium (RM)	MS salts and vitamins [49], 30 g/l sucrose, 7 g/l agar, pH 5.7.

Effect of agar concentrations on callus induction

Different concentrations of Agar (5g/l, 7g/l, 9g/l and 11g/l) were investigated for the maximum callus induction frequency. Mature seeds of the two cultivars that succeeded to induce callus were scored and also the explants that did not show any response or failed to keep alive were also scored.

Effect of plant growth regulators (Kinetin, NAA) on regeneration

This experiment was conducted to determine the level of Kinetin and NAA that most beneficial for rice regeneration. Calli of the two Egyptian rice cultivars were cultured on shoot regeneration medium (Table 1) supplemented with different concentrations of Kinetin (0, 1, 2 and 3mg/l) and NAA (0, 0.1, 0.2, and 0.3mg/l). The results were scored after four weeks. The responded calli that succeeded to from shoots were scored and also the calli that did not show any response or failed to keep alive were also scored.

Regeneration frequency

Regeneration efficiency of the two Egyptian rice cultivars, which includes the improvements discussed above, was determined. Regeneration frequency in this protocol is determined by establishing the ratio between the numbers of obtained regenerated shoots versus the number of callus used for regeneration.

Optical density (OD) of *Agrobacterium*

To maximize gene transfer rate of Egyptian rice cultivar Sakha104, five concentrations of *Agrobacterium* were employed at OD600= 0.2, 0.4, 0.6, 0.8, and 1 during the infection of Agrobacterium to callus. The results were scored after 3 weeks. The results were determined by the number of survival rice calli on selective medium (Table1).

Co-cultivation period

This experiment was carried out to determine the best co-cultivation period for transforming Egyptian rice calli Sakha104 with *Agrobacterium*. Different co-cultivation periods (1, 2, 3 and 4 days) were examined. The results were scored after 3 weeks. The results were determined by the number of survival rice calli on selective medium.

Dehydration treatment

The infected calli were air flowed in the laminar flow for twenty minutes. Then they were subjected to desiccation for 0,3,6,9 hours. The results were scored after 3 weeks. The results were determined by the number of survival rice calli on selective medium.

Co-cultivation temperature

In order to improve transformation efficiency of Egyptian rice cultivar Sakha104, Different co-cultivation temperatures (22, 25, and 28 °C) were tested. The results were scored after 3 weeks. The results were determined by the number of survival rice calli on selective medium.

Transformation procedure

Agrobacterium tumefaciens LBA4404 harboring the DREB2A gene (Figure 1) was grown on a shaker at 28°C in LB medium containing 50mg/l kanamycin and 50 mg/l rifampicin for 48hr until OD600 reached 0.6-0.8. The three week-old scutellacalli were soaked in *Agrobacterium* suspension for 10 minutes, blotted dry with sterile filter paper to remove excess bacteria. Then the calli were transferred to co-cultivation medium (Table 1) and incubated at 25°C under dark for 2-3 days. After co-cultivation, the infected calli were washed with sterilized distilled water containing 500mg/l cefotaxime to kill the *Agrobacterium* and then transferred to the selective medium.

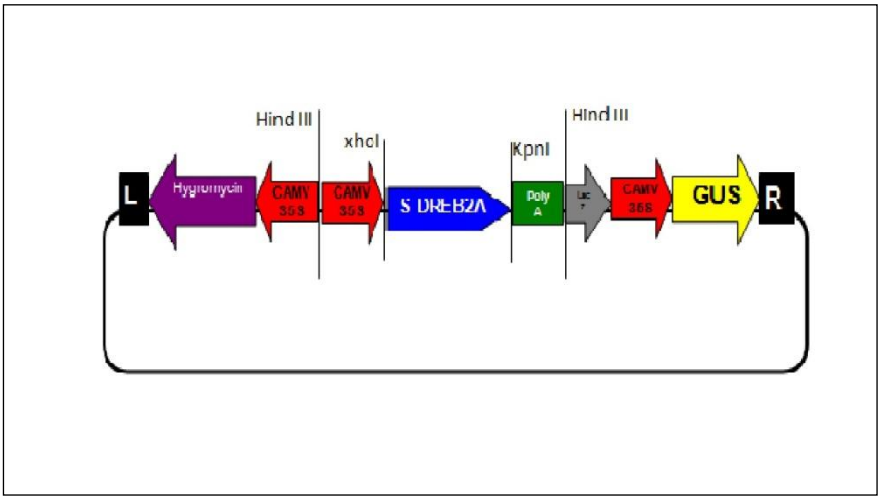


Figure 1. DREB2A Construct Map Vector

Selection and regeneration of transformant

After selection for 2-3 weeks, the surviving calli were transferred to the shoot regeneration medium containing 50 mg/l hygromycin for shoot regeneration. The regenerated shoots were further transferred to rooting medium (Table 1) containing 50 mg/l hygromycin for full plant formation with extensive root system.

GUS assay

GUS enzyme activity was assayed to determine the efficiency of the transformation process, according to the method described by Jefferson and his collegues[12]

Polymerase Chain Reaction (PCR)

Polymerase chine reaction was used to detect the presence of DREB2A gene in the transgenic tissues. Forward primer 5` gAgTACCTCgAgATggAgCgggggAggg 3` and Reverse primer 5` gCAgCggTACCgACTACTACTCTAATAggAg 3` were used for amplification. The PCR reaction was performed in 25 µl volume consisting of 2 µl genomic DNA (20-30ng), 2µl dNTPs (2mM), 2 µl of each primer (20µmol), 2µl 10x taq buffer , 1µl taq polymerase enzyme (promega, 5U\µl) and 14 µl dd H2O. PCR was initiated with one cycle at 94ºC for 4 minutes, followed by 33 cycles at 94ºC for 1 minute, 55ºC for 2 minutes, 72ºC for 1 minute and finally by one cycle at 72ºC for 10 minutes. After completion the cycles, the PCR products were resolved by agarose gel electrophoresis.

Statistical analysis

Experiments were setup in a randomized completely blocks designs (RCBD), twenty explants were used per treatment and each experiment was repeated three times. Means and standard errors (SE) were obtained from analysis for each treatment. Data were presented either as bar graphs with error bars by the use of computer program Microsoft Excel 2010 or as means ± SE.

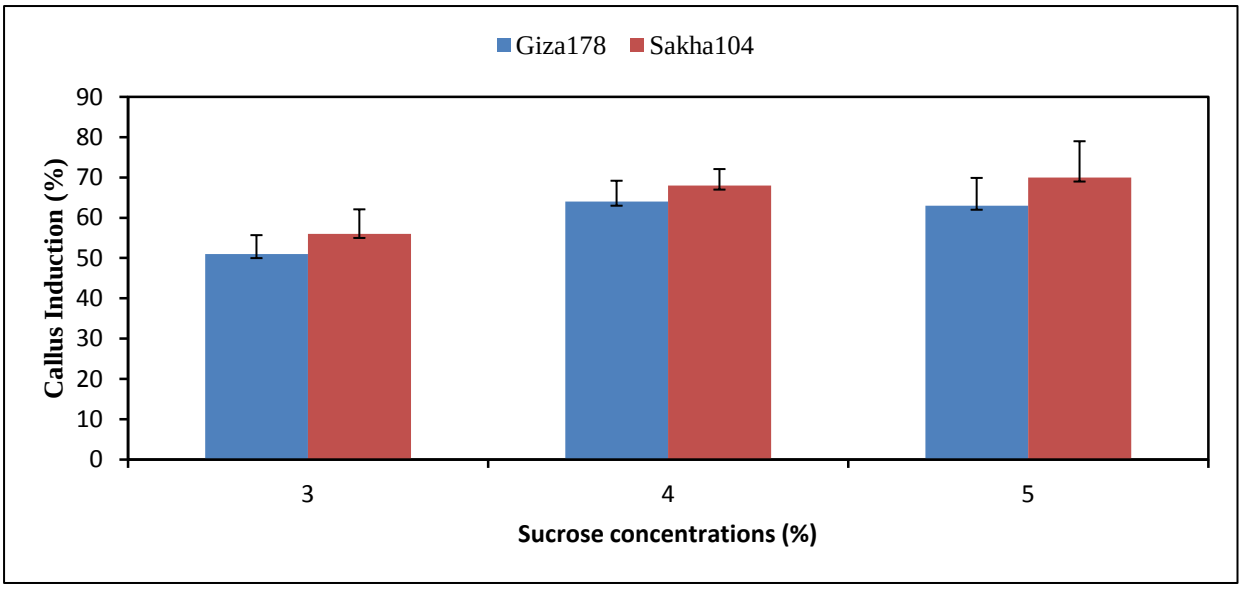
Results and Discussion

Rice regeneration

We selected two Egyptian rice cultivars i.e. Giza178 and Sakha104 for initial standardization of different factors critical for its enhanced regeneration and transformation. The basis of selection for these cultivars is their wide cultivation and high yield capacity. We used mature seed embryos as starting material for *in vitro* regeneration since they have distinct advantages over other explants such as their availability all round the year without restriction of season and geographical environment, and their easy operation and less infection by microorganisms[1].Moreover, embryonic calli obtained from mature seed embryos are efficient in rice transformation[13]. In the present study, mature seeds have developed scutellarcalli within14days of culture on our callus induction medium. We observed that scrutiny of embryogeniccalli from non-embryogenic ones was essential as this would affect the transformation and regeneration capability of the calli.

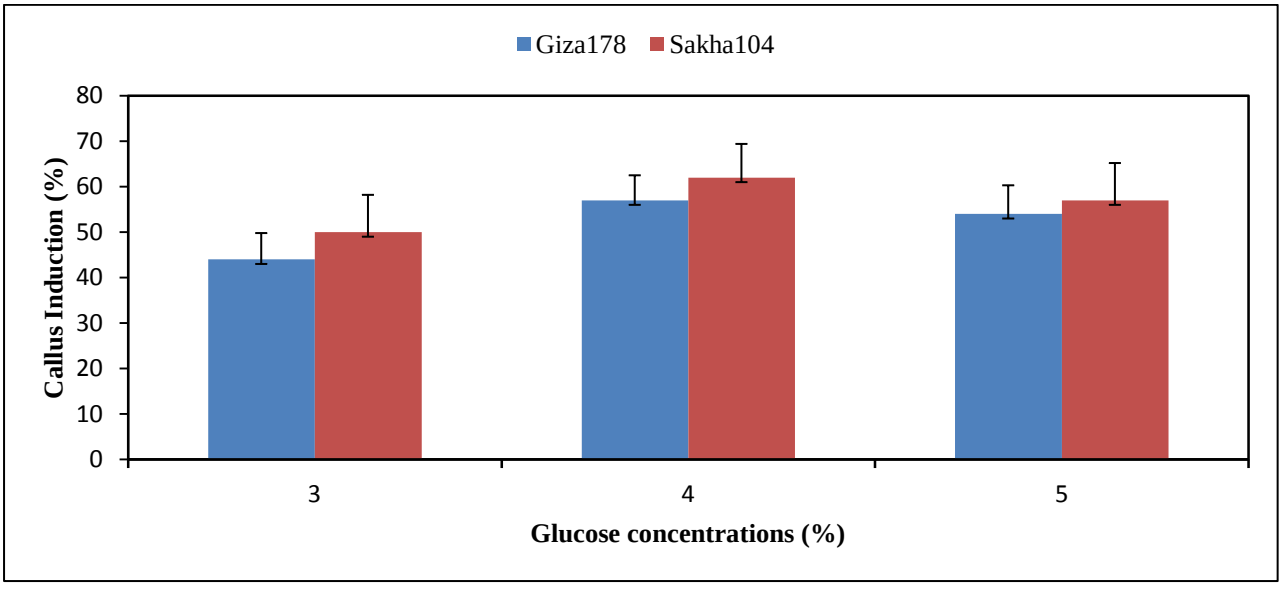
For efficient induction of embryogeniccalli from mature seeds of the two Egyptian rice cultivars, several types and concentrations of sugars were tested for their efficiency (Figure 2,3, 4).Based on these figures, The use of maltose was found to be the best followed by sucrose while the lowest frequency of callus induction was related to the use of glucose. 4% of maltose and glucose allocated the highest percentages of callus induction while there was no significant difference found between the use of 4% or 5% of sucrose. Plant, cell and tissue culture usually requires a carbohydrate source (sugar) in order to satisfy energy demands [14]. Sucrose has been widely used as the major carbohydrate source to supply energy to cells in plant tissue culture because of its efficiency in being transported across the plasma membrane [15]. Little attention has been given to the process of carbohydrate utilization *in vitro* or a comparison of the ability of different carbohydrates to support embryogenesis in cultured rice cells. The results presented in this work indicate the superiority of maltose to the other carbohydrates for callus induction and plant regeneration in rice. This finding confirms data ofRen and his collegues[16] who reported that the use of maltose significantly increased the frequency of callus formation in wheat. Recently,Sarojand his collegues[17] demonstrated that the addition of 4%maltose instead of sucrose into the culture media clearly increased the callus induction frequency in rice. They suggested that maltose may

protect the calli by reducing the production of ethylene since sucrose promotes *in vitro* production of ethylene in excised tissues causing the browning of callus. A color change in callus was observed in this study when higher than 4% sugar was used in the culture media. This might be explained by an excessive osmotic contribution or by the toxicity of the carbon source[18]. Therefore, maltose (at 4% concentration) was recommended to obtain the highest percentage of callus induction in Giza178 and Sakha104 rice cultivars.



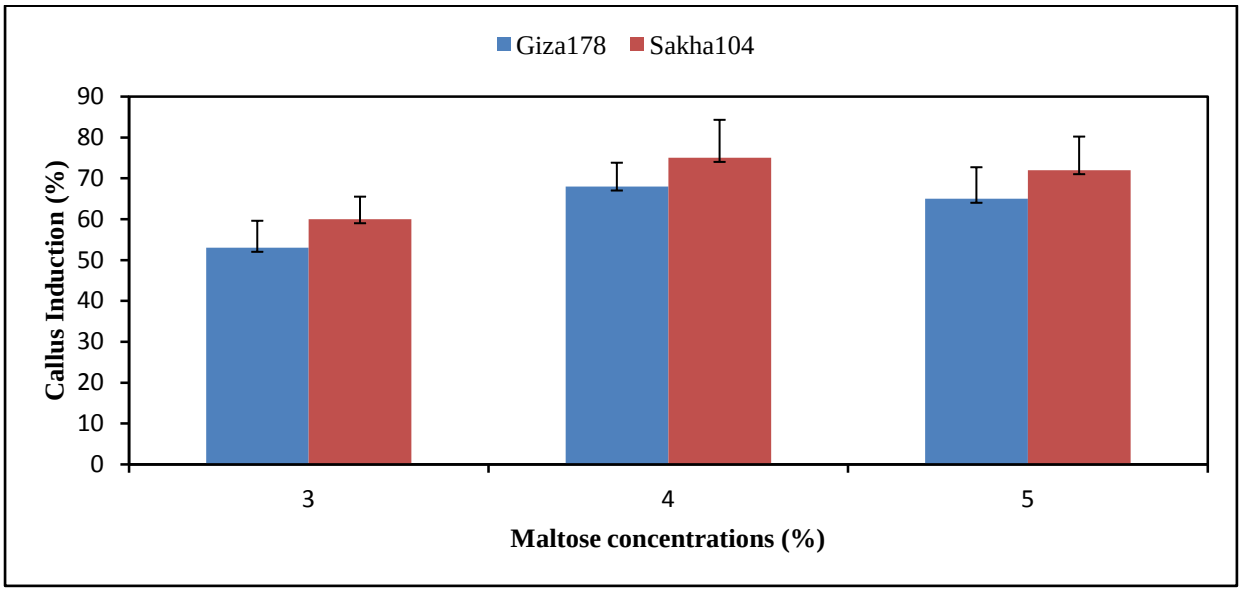
Values are means of three replicates ± standard errors.

Figure 2. Effect of sucrose concentration on callus induction percentage



Values are means of three replicates ± standard errors

Figure 3. Effect of glucose concentration on callus induction percentage



Values are means of three replicates ± standard errors

Figure 4. Effect of maltose concentration on callus induction percentage

Agar is the most commonly used gelling agent to solidify the media in plant tissue culture experiments. However, there are doubts about the inhibitory effect of agar on the growth and development of explants. Keeping in view this idea, we conducted a comparison among different agar concentrations (Table 2). In Sakha104 rice cultivar, it was observed that medium supplemented with 0.9% agar provided the highest frequency of callus induction (64%). However, there was no significant or remarkable difference between the most commonly used concentration of agar (0.7%) and 0.9% agar treatment. In addition, the same trend was also observed in Giza178 rice cultivar which behaved similarly as 0.9% agar concentration gave the highest callus induction percentage (60%) with no significant difference from 0.7% agar concentration(58%). Using a higher or lower concentration of the gelling agent, could promote callus induction but the frequency was lower as indicated in our experiments. In contrast to our data, some reports demonstrated that the supplementation of high level of agar (1%-1.2%) is efficient for the induction of embryogeniccalli in rice[17.]

Table 2. Effect of agar concentration on callus induction percentage

Agar concentration (g/l)	Callus induction (%)	
	Sakha104	**Giza178**
5	50 ± 3.9	50 ± 4.7
7	62 ± 5.1	58 ±3.8
9	64 ± 3.7	60 ±4.5
11	54 ± 4.5	48 ± 3.8

Experiments were setup in a randomized completely blocks designs (RCBD). Means and standard errors (SE) were obtained from analysis for each treatment.

Tissue culture media solidified with suitable gelling agent(s) offer appropriate milieu for the response of explants in order to proliferate and regenerate. Gelling agents provide optimum strength in order to support tissue responses hence, well prepared and solidified artificial media provide suitable conditions for the growth of plant cells and tissues. The mechanism governing the effects of gelling agents is complex. However, detailed studies indicate that the differential properties of various gelling agents depend upon the degree of clarity, polymerizability and water retention capacity, which in turn, influence mineral and carbohydrate availability [19, 20, 21]. It is accepted that choice of gelling agents and its concentration plays a predominant role in determining culture responses *in vitro*. In some systems a simple alteration of gelling agent may improve the culture of recalcitrant genotypes. Gelrite was found to enhance callus induction and *in vitro* regeneration in Swarna (89.9%) and Mahsuri (93.4%) rice varieties. Lin and Zhang[9] also have reported that 0.3% phytagel give higher callus induction. In the present study, it is evident that a relatively higher or lower concentration (0.5, and 1.1%)

than critical value (7-9%) caused an inhibition of callus induction. Thus, 0.7% agar was chosen as the optimum concentration in the culture medium for callus induction.

Table 3.Effect of plant growth regulators on shoot regeneration

Cultivar	Kinetin mg/l / NAA mg/l	0	1	2	3
Sakha 104	0	0	41±3.1	49±2.2	37±2.8
	0.1	0	56±2.7	61±1.8	44±2.1
	0.2	0	78±2.2	83±2.3	56±2.3
	0.3	0	71±2.6	81±2.1	64±1.9
Giza 178	0	0	34±3.3	42±1.7	31±2.5
	0.1	0	50±3.1	55±1.9	40±2.6
	0.2	0	71±2.8	76±2.1	51±2.9
	0.3	0	66±2.8	75±2.1	59±1.8

Experiments were setup in a randomized completely blocks designs (RCBD). Means and standard errors (SE) were obtained from analysis for each treatment.

Calli were transferred to media supplemented with different concentrations of kinetin and NAA for regeneration of shoots. Table (3) showed that calli transferred to medium supplemented only with NAA formed neither shoots nor plantlets. A combination of NAA with different concentration of kinetin led to the development of regeneration with variable response. In this respect, the highest frequency of shoot regeneration was observed on the MS medium supplemented with 0.2mg/l NAA and 2mg/l kinetin for Giza178 (76%) and Sakha104 (83%) cultivars. However, there was no significant difference between the uses of 0.2 mg/l or 0.3 mg/l NAA in combination with 2 mg/l kinetin. Plant hormones play very important roles in plant growth and development. The induction of callus and regeneration ability in plant tissue culture could be also regulated by plant hormones [22] .In 1957, Skoog and Miller discovered that auxin and cytokinin could regulate morphogenesis and plant development, as they put forward the hypothesis of hormone balance [23]. The present study has also demonstrated that the auxin to cytokinin ratio is decisive to *in vitro* response of plant tissues. Low amount of NAA in combination with relatively high amount of kinetin provided the best frequency of shoot regeneration. Exposing callus cultures to a high ratios of auxin:cytokinin resulted in root formation, whereas, a low ratio promoted shoot regeneration. It was reported that hormonal metabolisms operated in an integrated manner [24] and that several, potential, mutual functional interaction points exist between different hormones [25]. The synergic or antagonistic effects among plant growth regulators could explain why the use of multiple hormones versus a single hormone was more effective in plant regeneration. This explains why the addition of NAA clearly increases the efficiency of kinetin for shoot regeneration in our experiments.

Table 4. Regeneration frequency of Giza178 and Sakha104 cultivars

Cultivar	Callus induction%	Regeneration %
Giza 178	72 ±2.3	76±1.8
Sakha 104	79±2.1	83±1.9

Experiments were setup in a randomized completely blocks designs (RCBD).Means and standard errors (SE) were obtained from analysis for each treatment.

We employed all the above studied factors to obtain the maximum regeneration frequency for the two Egyptian cultivars G178 and Sakha104.Clear differences in the regeneration frequency were observed among the two examined rice cultivars (Table 4). Sakha104 proved to have the highest callus induction and regeneration frequency whereas Giza178 had lower frequency. Since Sakha104 cultivar showed the highest regeneration capacity among the two tested cultivars, it was selected for further transformation experiments. It was obvious that regeneration frequency of the two Egyptian rice cultivars is depending on the genetic

background. The different genotypes had different sensitivity to the regeneration frequency. This behavior is in accordance with that of Nishimura and his collegues[26] who pointed out that the regeneration process had to be genotype independent. It is generally held that genotypes remain the major limiting factor restricting successful transformation in rice varieties. Although a dozen of optimized tissue culture media for rice were published in literature, they are largely genotype-dependent[9]. The obtained data represent a high percentage of callus induction and regeneration for the Egyptian rice cultivars. El-Shawaf and his collegues[27] studied the efficiency of five Egyptian rice cultivars including Sakha104. They obtained a regeneration frequency as high as 70.7% for Sakha104. It is worthy to say that our improvements clearly increased the regeneration frequency. Therefore, the protocol reported here is suitable for genetic transformation due to its high efficiency, reproducibility and the easy accessibility of mature seeds.

Rice transformation

Transformation of rice has been attempted by many authors using different approaches, i.e., DNA uptake[28], electroporation[29], particle bombardment[30] and the *Agrobacteriumtumefaciens* method[31]. Nevertheless, 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, integration of a small number of copies of the gene and more importantly, the possibility that even large segments of DNA can be efficiently transferred [32]. Various parameters for the maximum gene transfer rates of Egyptian rice cultivar Sakha104 using *Agrobacterium tumefaciens* method were optimized. Also, we reported on the introduction of DREB2A gene driven by the CAMV35s promoter into rice as the first step to increase the high temperature tolerance characteristic of the crop.

Table 5. Effect of *Agrobacterium* concentration on transformation efficiency

***Agrobacterium* concentration(OD600)**	**hygromycin-resistant calli %**
0.2	0
0.4	16±2.1
0.6	21±2.7
0.8	22±3.2
1	14±2.9

Experiments were setup in a randomized completely blocks designs (RCBD). Means and standard errors (SE) were obtained from analysis for each treatment.

Optical density (OD) of *Agrobacterium* strain is an important factor for genetic transformation in rice. In this study, different concentrations of *Agrobacterium* (OD600 = 0.2, 0.4, 0.6, 0.8, 1) were tested as shown in table (5). It was observed that the increase of *Agrobacterium* concentration was accompanied by parallel increase in the percentage of survival rice calli. No transformation event was detected on the lower bacterial density (OD600=O.2). All calli could not survive on medium contained hygromycin indicating the failure of the transformation process. The calli showed remarkable sensitivity with OD600=0.4 and the survival percentage of calli was 16%, whereas calli showed a slight sensitivity and the survival percentage was 21% when OD600=0.6. The highest efficiency of transformation was detected when OD600=0.8.However, Increasing the *Agrobacterium* concentration to OD600=1 decreased the frequency of transformation. Zhang and sompong[33] showed that OD600=0.6 was the best *Agrobacterium* concentration for efficient transformation in rice. Previous reports indicated that the OD600 of the bacterial density in a range from 0.3 to 1 is suitable for transformation [34],[35].In the present study, OD600=0.6 to 0.8 proved to be the best concentration of *Agrobacterium* for successful transformation in Egyptian rice cultivar shaka104.

Table 6. Influence of co-cultivation period on transformation efficiency

Co-cultivation period (days)	**hygromcin-resistant calli %**
1	0
2	17±1.4
3	20±2.1
4	13±1.3

Experiments were setup in a randomized completely blocks designs (RCBD). Means and standard errors (SE) were obtained from analysis for each treatment.

Different co-cultivation periods (1, 2, 3 and 4 days) were investigated. The infected calli for one day did not show any transformation efficiency (Table 6). Two and three days co-cultivation periods showed the highest percentage of transformation efficiency (17%, 20%, respectively) whereas the transformation frequency was decreased dramatically in the case of using 4 days as a co-cultivation period (13%). Therefore, subsequent experiments will use a 3- day co cultivation period as prolonged exposure of cells to *Agrobacterium* may adversely affect the calli and cause cell death or overgrowth of bacteria. The efficiency of *Agrobacterium* infection differed by explants type and co-cultivation period. For example, the optimal co-cultivation time for banana suspension cell cultures was 7 days[36], 3 days for embryogenic rice suspension cells[37], and 2 to 3 days for rice callus [38]. In the present study, a 3- day co-cultivation time resulted in the highest transformation efficiency without overgrowth of *Agrobacterium* as longer time will cause bacterial overgrowth. These results are in agreement with those of Bernal and his collegues[39] who used 3 days of co-cultivation period and obtained improved transformation frequency compared to 5 days of co cultivation period.

Transformation frequencies during co-cultivation at 22, 25 and 28°C were compared. Interestingly, the highest transformation frequency was obtained at 25°C, followed by that at 28°C while the lowest transformation frequency was obtained at 22 °C (Table 7). The temperature of co-cultivation is reported to be a critical factor to the efficiency of genetic transformation mediated by *Agrobacterium*[40]. Most reports in rice transformation employed 28°C as the best co-cultivation temperature[41,42]. We observed significant differences in efficiency among co-cultivation temperatures of 22, 25 and 28 °C, perhaps reflecting the sensitivity of our cultivar. We reported for the first time -up to our knowledge- that the transformation efficiency in rice could be maximized by decreasing the co-cultivation temperature from 28 °C to 25°C. It should be noted that lower co-cultivation temperatures have been previously reported to increase transformation efficiency in other plant species such as soybean[43]and tobacco[44]. However, such a result needs to be investigated and confirmed in further studies.

Table 7. Influence of co-cultivation temperature on transformation frequency

Temperature (C°)	hygromycin-resistant %
22	18±2.1
25	24±1.6
28	20±1.3

Experiments were setup in a randomized completely blocks designs (RCBD). Means and standard errors (SE) were obtained from analysis for each treatment.

Table 8. Effect of Dehydration treatment on transformation efficiency

Dehydration treatment %	hygromycin-resistant calli
0	20 ±3.2
3	19±3.8
6	18±4.1
9	21±3.3

Experiments were setup in a randomized completely blocks designs (RCBD). Means and standard errors (SE) were obtained from analysis for each treatment.

Different dehydration treatments were investigated in order to maximize the gene transfer rate of Egyptian rice. Table (8) showed that there were no significant differences between desiccation treatments. Moreover, there was no significant difference between 0hour desiccation (without desiccation) and desiccation treatments. Our results are in contrast to Gang and his collegues[45] who indicated that the dehydration treatment improved the regeneration of transformed rice calli. Their results might be due to their different method of drying as they dehydrated rice calli many times and for a longer period. It also might be explained by the different cultivars that we used in our experiments (Egyptian cultivars).

Histochemical GUS assay was carried out to assess the expression of GUS gene in the transformed calli. The hygromycin resistance calli showed blue color indicating GUS activity, confirming GUS expression,

while the non-transgenic calli did not show any GUS activity (Fig. 5).Analogous results were reported by many researchers such as Saker and his collegues[30] , Ozawa[46] and Wagiran and his collegues[31] who performed histochemical GUS assay to indicate the efficiency of their transformation protocols. The GUS gene has been the most widely used reporter gene for the analysis of plant gene expression in plant transformation systems as described by Jefferson and his collegues[12]. The GUS expression system has been further improved by cloning an intron within the GUS gene region, which completely inhibits its expression in *Agrobacterium*. Thus, allowing the precise visualization of transformation events at early stages after co-cultivation. In the present study, the improved GUS expression system was used to confirm the stable integration and expression of GUS gene into rice genome.

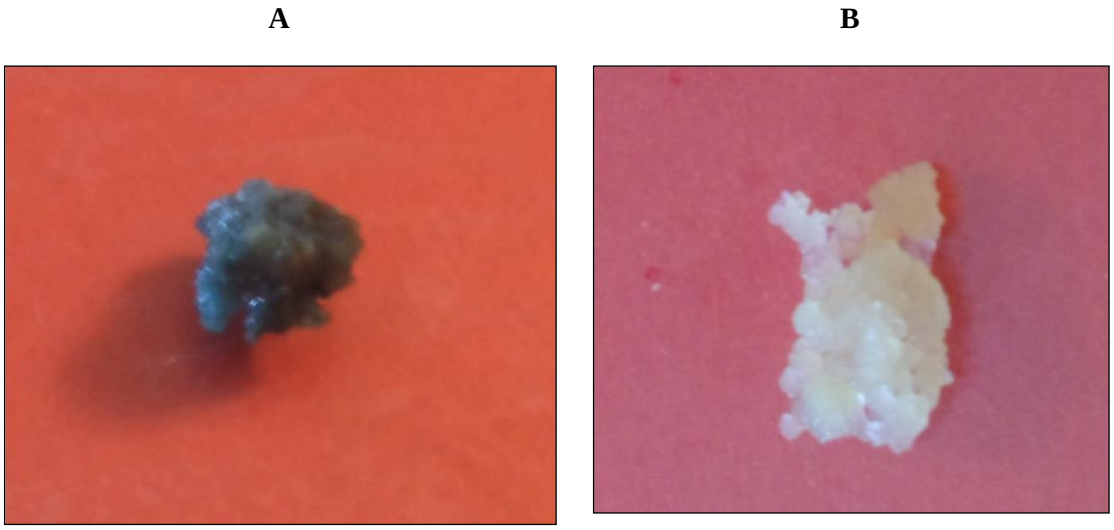


Figure 5.Histochemical GUS assay in rice. A: Gus expressing calli. B: non transgenic callus

PCR is a sensitive technique allowing single-copy genes to be amplified and extracted out of a complex mixture of genomic sequences. Amplified DNA is visualized as distinct bands on agarose gels. Therefore, PCR was utilized to confirm the integration of T-DNA fragments in hygromycin-resistant plantlets. Leaves of putative transgenic plantlets were analyzed using PCR with primers designed to amplify the relevant sequence of DREB2A gene. The obtained results illustrated in figure (6) confirmed the presence of the expected 825 base pairs fragment. Using the polymerase chain reaction technique as indicator for the presence of transgenes in the genomic DNA of putatively transgenic plants has been reported by many investigators such as, Wagiran and his colegues[31] who used PCR to evaluate transformed rice plants resulted from *Agrobacterium*-mediated transformation protocol in Japonica rice suspension cell culture.

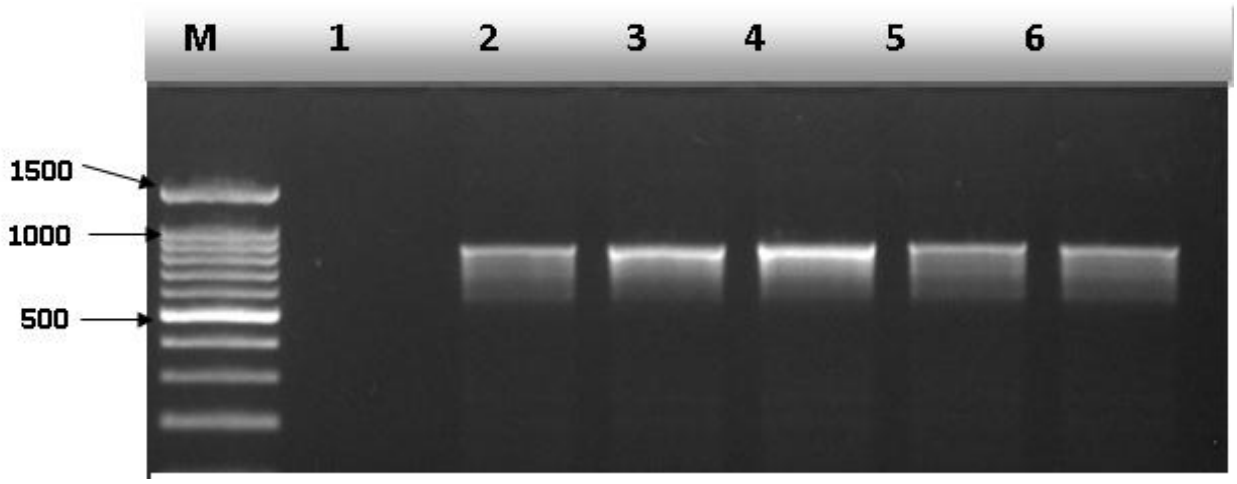


Figure 6.Molecular confirmation of the integration of the T-DNA into plant genome by PCR using DREB2A- specific primers. Lane 1: negative control, lane 2: positive control and lanes 3-6:putative transgenic plants

In this study, we investigated some valuable clues affecting regeneration and transformation frequency. An efficient system for regenerating transformed Egyptian rice with DREB2A gene was obtained. Our procedure permitted the production of this transgenic rice in only three months. This effort is the first step in creating new Egyptian rice cultivar tolerant to heat stress.It is hoped that our improved protocol will be integrated in Egyptian rice breeding programs for improving local rice cultivars with other traits as well.

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