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Genetic transformation of white poplar (*Populus alba* L.) with glutaredoxin-2 gene

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Genetic transformation of an elite white poplar genotype (*Populus alba* L.) was performed with vector pRI 101-ON DNA in *Agrobacterium tumefaciens* strain LBA4404 containing the Glutaredoxin-2 gene in order to enhance the resistance to abiotic harmful stress. The time periods of shoot tips infection with *Agrobacterium tumefaciens* suspension culture (0, 5, 10, 20 and 30 min) was tested growth parameters at all stages growth for the transformation frequency. The highest frequency of transformation was obtained with shoot tip explants infected with *Agrobacterium* culture for 20 minutes at the concentration of OD_{600}= 0.8. Isoenzymes peroxidase and polyphenyl oxidase were used as indicators for GRX2 expression, formed transgenic poplars had highly total peroxidase and polyphenyl oxidase expression compared with that of the control.

Keywords: *Agrobacterium*, *Populus alba*, Glutardeoxin 2, peroxidase, polyphenyl oxidase

INTRODUCTION

Poplars are deciduous trees of the willow family (*Salicaceae*) with over 30 species distributed in the temperate areas of the Northern hemisphere that differ in leaf size and shape as well as in the color of the bark, etc. They have a short life span but are one of the fastest growing temperate trees – they can grow 5–10 feet per year (Stanturf et al. 2001 and Tuskan et al. 2006).

White poplar (*Populus alba* L.), of section *Populus* (syn. Leuce Duby), is a native to the Mediterranean basin being widely distributed in floodplain forests throughout northern Africa, southern Europe, and Central Asia (Brundu et al. 2008). *Populus* species are grown worldwide for forest products, such as pulp fiber, dimension lumber, furniture components, flakes for oriented strand board (OSB), and veneer, and are now being proposed as a bioenergy crop (Davis, 2008). Rutledge and Douglas (1988) tested regeneration of poplar plants from meristem tips to avoid virus contamination

The poplar is an important model for woody perennial biotechnology because it is amenable to *in vitro* culture and genetic engineering through *Agrobacterium*-mediated transformation (Confalonieri et al. 2000; Han et al. 2000; Dai et al. 2003), *Populus* a suitable model system for genetic engineering of deciduous trees. (Kim et al. 1997.Glutaredoxins (Grxs) are glutathione-dependent thiol disulfide oxidoreductases of the thioredoxin family present in all organisms from bacteria to human (Noguera et al. 2005).

High concentrations of glutathione along with high GSH/GSSG redox potential has been suggested to confer better antioxidative protection and might be considered an acclimation against the stress factor (Tausz et al. 2004). Grxs play important biological functions in plants, such as oxidative stress responses, iron-sulfur (FeS)

cluster assembly, and cell signaling, etc. (Rouhier et al, 2008). In plant cell, antioxidant enzymes such as polyphenyl oxidase (PPO), peroxidase (POD) and catalase (CAT) have been considered as defensive team, whose combined purpose is to protect cells from oxidative damage These findings suggest that the induction of reactive oxygen species (ROS) scavenging enzymes, such as PPO, POD and CAT, is the most common mechanism of salt tolerance for detoxifying ROS synthesized (Gao, 2008 and Koca et al. 2007).

The aim of this research work is to establish a protocol to transfer the salinity tolerant gene (Glutaredoxin-2) into *P. alba* by *Agrobacterium*-mediated transformation, in order to improve its abiotic stress tolerance. The development of transgenic *P. alba* with salt-tolerant properties will largely extend cultivated areas for planting *P. alba* and improve the utilization efficiency of saline water.

MATERIALS AND METHODS

This research work was achieved in Plant Tissue Culture Laboratory and Plant Biotechnology Research Laboratory, Horticulture Research Institute (H.R.I.), Agricultural Research Center (A.R.C.), Giza, Egypt, during the successive years 2012-2016.

Agrobacterium cultur and transformation strain

Agrobacterium tumefaciens strain LBA 4404 harboring a binary vector (pRI 101 – on DNA – Grx2) was used for plant transformation experiment which was introduced into *A. tumefaciens* according to the method of Tzfira *et al*. (1997). The binary vector consisted of the Glutaredoxin-2 target gene (Grx-2), which was cloned from *Cyan bacterium synechocystis* PCC 6803, under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter and nopaline synthase (*nos*) terminator, and the selective Kanamycin resistance gene npt II.

Plant Material

Plant material was collected from a *Populus alba* mother tree, located at Gemmeiza Agric., Research Station, Kafr El Sheikh Governorate, Timber Trees Dept., Horticulture Research Institute, Agricultural Research Center, Egypt. Adventitious shoot regeneration of *P. alba* were achieved as described by (Gad, 2011).

Populus alba transformation

Explants were basely trimmed and ~1.0 cm vertically cultured on MS- medium (Murashig and Skoog, 1962), (MS + 30 g/l sucrose + 0.6 mg/l BA + 1.0 mg/l GA_3+ 7 g/l agar) then were cultured in glass jars, culture explants were incubated for 4 weeks under controlled conditions, Shoot tips of proliferated shootlets from *P. alba* cultured explants were basely wounded and subjected to transformation experiment, immersed in the bacterial suspension in Petri dishes and inoculated for different periods (0, 5, 10, 20 and 30 min) with gentle agitation to increase the transformation rate shoot tips were then dried on sterile filter paper and cultured on regeneration MS medium (MS + 30 g/l sucrose + 0.6 mg/l BA + 1.0 mg/l GA_3+ 7 g/l agar) supplemented with 500 mg/l cefotaxime, and incubated in the growth room for 2-3 days in darkness without Kanamycin sulfate (Kan). After 2-3 days, shoot tips were transferred on the same fresh regeneration medium (MS + 30 g/l sucrose + 0.6 mg/l BA + 1.0 mg/l GA_3+ 7 g/l agar) supplemented with 100 mg/L Kanamycine and 500 mg/L cefotaxim for selection, then incubated at 25 °C under 16 h/day photoperiod of 40 µE cm $^{-2}S^{-1}$ for one month. Survived shootlets were separated and transferred to MS medium supplemented with (MS + 30 g/l sucrose + 1.4 mg/l BA + 1.0 mg/l GA_3+ 7 g/l agar) for 4 weeks under the same controlled condition, multiple shoots tips regenerated on this medium were than separated and sub-culture in the same medium every 2 weeks for further proliferation, once shoot were obtained, they were transferred to the shoot elongation medium. Shootlets of 2-3 cm long were separated and sub-culture after 8-12 weeks and placed on MS elongation medium containing (MS + 30 g/l sucrose + 0.5 mg/l BA + 3.0 mg/l GA_3 + 3 g activated charcoal + 7 g/l agar) medium containing Kan. and Cef. The shoots were then transferred to rooting medium (MS + 30 g/l sucrose + 0.5 mg/l IBA + 3.0 mg/l GA_3+0.1 mg/l NAA+ 3 g activated charcoal + 7g/l agar) after regeneration selection. The transformation efficiency based on the effect of inoculation period with *A. tumefaciens* on *P. alba* different characters, calculated using diagnosis of DNA PCR results, as well as Isozymes analysis. Vegetative growth was based on survival percentage, number of shootlets/explant, shootlets height, number of leaves/ explant, , root length of plantlets from successfully transformed plantlets. *Agrobacterium* transformation and regeneration of putative transformation various

factors affecting the transformation efficiency: infection duration time was tested in this study.

DNA isolation and PCR

Fresh leaves samples were collected from putatively transformed and non-transformed (control) plants. The DNA extraction was performed using DNeasy plant Mini Kit (QIAGEN). Genomic of extracted DNA was analyzed by PCR, polymerase chain reaction (PCR) analysis was performed on the isolated DNA to amplify a 281 bp fragment of the GRX2 by using specific primers, **ssr 2061 F (5/ AAGCGTTCATATGGCTGT CT 3/**), and **ssr 2061 R** (**5/ CTAACTATGGAGCAGGGGGT3/).**PCR amplification was performed as described above (the PCR program to GRX-2 gene) . The DNA bands were visualized on ultraviolet (UV) Trans illuminator and photographed with gel documentation system. *P*Grx2 was used as a positive control template while the wild-type genomic DNA obtained from species served as a negative control. The PCR reaction was performed in a 20 µl reaction mixture containing 40 ng DNA templates, 10 pmole of each forward and reverse primers, 10 µl of 2X power Taq PCR master mix (Biotecke Corporation) and volume was completed to 20 µl using sterilized distilled water. The PCR program were as follows: 94 ºC for 5 min followed by 35 cycles of 94 ºC for 1 min, 58 ºC for 1 min and 72 ºC for 1 min, and a final 7 min extension at 72 ºC

Antioxidant Isoenzymes

Isozymes electrophoresis native polyacrylamide gel electrophoresis (Native - PAGE) was conducted to identify isozyme variations among transformed and non-transformed studied plants using two isozyme systems,

1- Polyphenyl oxidase isoenzmes (EC. 1.10.3.1) Leaves samples (100 mg fresh weight) was determined by the method of (Thipyapong et al. 1995 and Bradford, 1976).

2- Peroxidase isoenzmes (EC. 1.11.1.7) POD was determined by the procedure described by Ros Barcelo et al. (1987).

Statistical analysis

Data obtained were statistically anayzed and mean separation was made by using least significant differences (L.S.D.) at 5 % level of significance. Test was applied for the comparison among means as described by Steel and Torrie (1980)

RESULTS

Effect of *P. alba* infection period with *Agrobacterium tumefaciens* on vegetative growth

Explant survival (%):

Data in Table (1) represent that the infection period for 20 min of shootlets grown on selective medium with *Agrobacterium tumefaciens* got a high survival percent value (85.7 %), however, shoot tips infected for 30 min produced the lowest survival (28.6%). The control shoot tip that had grown on non-selective medium completely survived 100%, with no significant difference between control and 20 min period of inoculation.

Number of shootlets/explant

Data shown in Table (1) indicate that the infected shoot tips for 20 min with *A. tumefaciens* got the highest value of shootlets number per explant (1.43), while the three different infection periods (5, 10, 30) min produced the same value for shootlets number (1) while non-infected shoot tips (control) produced (1.86) shootlets/explant.

Shootlets height (cm)

Concerning to shoot tips cultured on the selective medium, data in Table (1) reveal that the infection period for 20 min with *Agrobacterium tumefaciens* resulted in the highest shootlets length (2.39 cm). Meanwhile, shoot tips infected for 30 min had the shortest shootlets (0.57 cm) compared to non-infected control shootlets (2.22 cm).

Number of leaves / shootlet

Data represented in Table (3) reveal that infection of shoot tips for 20 min with *A. tumefaciens* got the highest value of leaves number (13.86) on the other hand, shoot tips infected for 30 min produced the lowest number of leaves (5.71), while control of non-infected explants and has grown on non-selective medium produced (12.71) leaves.

Table (1). Effect of different inoculation periods (0.0, 5, 10, 20 and 30 min) with *A. tumefaciens* on vegetative growth of *Populus alba* during establishment stage

Period of inoculation (min)	shootlets survival (%)	shootlets height (cm)	Leaves no/ shootlet
0.0	100.0	2.22	12.71
5.0	57.1	1.07	7.00
10.0	71.4	1.32	8.00
20.0	85.7	2.93	13.86
30.0	28.6	0.57	5.71
LSD (0.05)	46.4	0.51	4.33

Shootlets elongation

During multiplication stage, shootlets were stunted and trials to obtain rooted shootlets with fully expanded leaves directly through rooting stage were failed. Therefore it was necessary to pass through shootlets elongation stage with true leaves through medium modification with activated charcoal ($3gl^{-1}$ AC) and gibberellic acid ($3\ mgl^{-1}$ GA_3) ,which played their enhancing role, regardless to their combination with BAP with or without $0.1mgl^{-1}$ NAA. In this stage, explant survival (%), plant height and leaves number/shootlet were subjected to the influence of different periods of infection with *A. tumefaciens* (0, 5, 10, 20, 30) min. Shootlets originated from shoot tips grown on selective medium compared with control shootlets grown on non selective medium after four months of inoculation, as exhibited in Table (2).

Shootlets survival (%):

Data in Table (2) represent that shootlets grown on selective medium with infection period for 20 min with *A. tumefaciens* got high survival percent value (52.7 %). However, explants infected for 5 min induced the lowest survival (23.4%). Elongated shootlets produced from control shoot tip that had grown on nonselective medium completely survived (100%).

Shootlets produced from shoot tips infected with *A*. *tumefaciens* suspension culture for 30 min. were died before elongation stage.

Shootlets height (cm)

Concerning to elongated shootlets produced from shoot tips cultured on the selective medium, data indicate that the infection period for 20 min. with *Agrobacterium tumefaciens* resulted in the highest shootlets length (4.2 cm). Meanwhile, shootlets produced from shoot tips infected for 5 min had the shortest shootlets (3.03 cm) compared to non-infected control shootlets (4.5 cm), as indicated in Table (2).

Number of leaves / shootlet

Data represented in Table (2) reveal that elongated shootlets produced from infected shoot tips for 20 min with *A. tumefaciens* got the highest value of leaves number (9.57). On the other hand, shootlets from shoot tips infected for 5 min produced the lowest number of leaves (5.43), while control of non-infected explants that it has grown on non-selective medium produced (12) leaves.

Plantlets rooting

During rooting stage, elongated plantlets were subjected to the extended effect of different periods of shoot tips inoculation (5, 10 and 20) min. compared with the control plantlets. Survival (%), plantlets height and root length were determined.

Plantlet height (cm)

It is quite evident generally that the infection of shoot tips for 20 min with *Agrobacterium tumefaciens* got the highest value of length (11.9) cm. On the other hand, shoot tips infected for 5 min produced the lowest planlets height of length (8.90) cm, while the control planletes height was (10.4) cm, as revealed in Table (3).

Umber of leaves / plantlet

Data represented in Table (3) reveal that infection of shoot tips for 20 min with *Agrobacterium tumefaciens* got the highest value of leaves number (8.53). On the other hand, shoot tips infected for 5 min produced the lowest number of leaves (5.8), while control non-infected explants that it has grown on non-selective medium produced (10.31) leaves per rooted planlet.

Root length (cm)

Concerning to shoot tips cultured on the selective medium, data indicated that the infection period for 20 min with *A. tumefaciens* resulted in the highest root length (7.62 cm).

Table (2) Extended effect of different inoculation periods (0.0 (control), 5, 10, 20 and 30 min) with *A. tumefaciens* on vegetative growth of *Populus Alba* during elongation stage.

Period of inoculation (min)	shootlets survival (%)	shootlets height (cm)	Leaves no/ shootlet
0.0	100.0	4.50	12.00
5.0	23.4	3.03	5.43
10.0	34.1	3.68	8.83
20.0	52.7	4.26	9.57
LSD (0.05)	55.1	0.45	2.31

Table (3).Extended effect of different inoculation periods (0.0, 5, 10 and 20 min.) with *A. tumefaciens* on vegetative growth of *Populus alba* plantlets during rooting stage.

Period of inoculation (min)	Plantlet height (cm)	Leaves no./ plantlet	Root length (cm)
0.0	10.4	10.31	6.56
5.0	8.90	5.80	6.05
10.0	9.42	7.76	7.40
20.0	11.90	8.53	7.62
LSD (0.05)	4.00	2.35	2.04

Meanwhile, shoot tips infected for 5 min had the shortest roots (6.05 cm) compared to non-infected control plantlets (6.56 cm).

The data indicated that explants shoot tips used stable for poplar regeneration this agreement with Nadel et al. (1992), that it inoculated with *A.tumefaciens* suspension (OD_{600} = 0.8) for 20 min. and grown in selective medium which contain 100 mg/l (kan) got the highly transformation frequency, similarly with previous studies by (Confalonieri et al. 2000, Bansal et al. 2008, Han et al. 2013 and Movahedi et al. 2014). Transformation efficiency might also be influenced by the co cultivation time between explants and *Agrobacterium* according to a study (Rutledge and Douglas, 1988 and De Block 1990). The explants that were pre-cultured for 3 days survived the infection better accelerates the rate of T-DNA incorporation in cells by increasing their competence rather than increasing the rate of DNA transfer from *Agrobacterium*, allowing a greater survival of transformed cells that it agreement with (Binns and Thomashow, 1988). Similar findings were observed in a variety of cottonwood species (De Block, 1990 and Balestrazzi et al, 2000; Han et al, 2000).

Integration and expression of GRX-2 gene in transgenic *P. alba* shootlets

Wounded shoot tips and infected with a single cell culture of *A. tumefaciens* strain LBA 4404 carrying the GRX-2 and nptII genes in the pRI 101-ON DNA plasmid vector, produce adventitious shootlets which were selected on selective medium congaing kanamycine as a selective agent. Shoolets that regenerated from these shoots were selected as putative transgenic plants.

Molecular Analysis of Putative Transformed plantlets

Randomly chosen putative transformed *P. alba* plantlets subjected to different periods of infection (5, 10, 20, 30) min were subjected to PCR based assay to detect the presence of the Grx-2 gene and the non-transgenic plant as the negative control. The presence of the expected 281 bp fragment after PCR amplification indicated the presence of the transgene in the regenerated plants (Fig. 1). The plasmid was used as a positive control (+C). No amplification of the expected size was observed in the non-transgenic control plants (– C).

Antioxidant isozymes.

Isozymes merely represent different structure configuration of the same polypeptide chain of an enzyme (**Morsy, 2007**). For this reason two isozymes including polyphenyl oxidase (ppo) and peroxidase (px) were used to study the effect of different inoculation periods with *A. tumefaciens* on the gene/genes expression for *P. alba* plantlets.

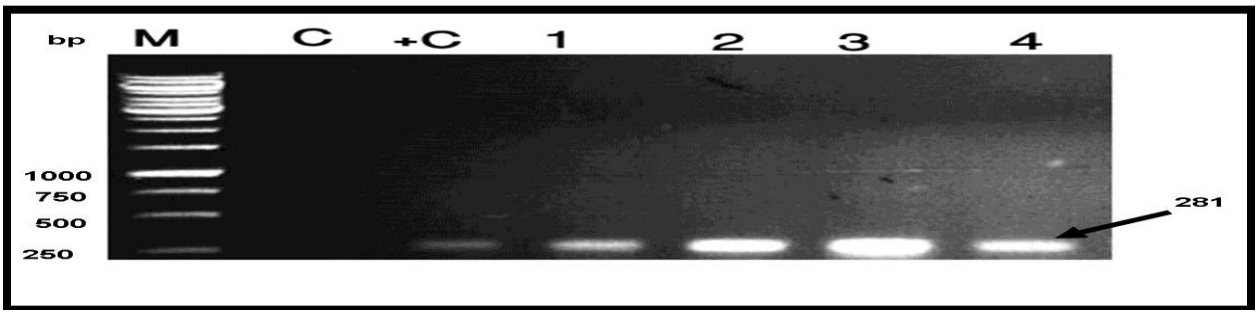

Figure.(1): PCR analysis of genomic DNA isolated from leaves of non-transformed control and transgenic *P. alba* shootlets using primer pairs specific for amplification of GRX-2 gene. M: Molecular DNA marker (1kb); (-C): negative control (non-transgenic); (+C): positive control (plasmid); and lanes 1-4: different transgenic *P. alba* plants.

Table (4): Polyphenyl oxidase isozymes ideogram analysis of *P. alba* plantlets subjected to different periods of inoculation with *A. tumeficens* (Lane 1: 0.0 min, Lanes (2:4) 5, 10 and 20 min.) respectively.

RF	Lane 1	Lane 2	Lane 3	Lane 4
0.092	–	-	+	+
0.233	–	-	+	+
0.837	–	++	++	++
0.972	++	++	++	+++

– : absent, + : low density, ++ : moderate density and +++ : high density

Table (5): Peroxidase isozymes ideogram analysis of *P. alba* plantlets subjected to different periods of inoculation with *A. tumeficens* (Lane 1: 0.0 min, Lanes (2:4) 5, 10 and 20 min.) respectively.

RF	Lane1	Lane2	Lane3	Lane4
0.166	+	–	+++	+++
0.600	–	–	++	++
0.749	++	++	++	++
0.902	++	++	++	+++

(–: absent, +: low density, ++: moderate density and +++: high density)

Polyphenyl oxidase isoenzmes (EC. 1.10.3.1)

Polyphenyl oxidase electrophoretic patterens in Fig. (2) and Table (4) exhibit a maximum of 4 consisted bands very well visible on the gel though tightly associated with relative mobility range from 0.092 to 0. 972 and characterized by a clear inter and intra polymorphism in bands presence and absence and variable densities among different periods inoculation with *A. tumefaciens* and control samples. Shootlets which were infected with *A. tumefaciens* for 20 min and 10 min produced 4 bands with relative mobility (0.972, 0.837, 0.233 and 0.092) and different densities whereas the band at (Rf 0.972) had higher density in inoculation for 20 min while inoculation for 10 min. produced moderate density. The inoculation for 5 min produced two moderate bands at (Rf 0.837 and 0.972), though, control plant produced just one band at (Rf 0.972) with moderate density. The results indicated that the plants inoculated with *A. tumeficen*s for 20 min their gene expression was highly activity representative in comparison with other different periods of inoculation and the control plant.

These results may be explained on the basis that the appearance and disappearance of isozymes group bands and different densities for each band is related to the effect of GRX2 gene expression that it happen as a resulted for stress on transformation of plants accordance with Barbehenn et al. (2007).

Peroxidase isozyme (EC. 1.11.1.7)

Peroxidase electrophoretic patterens in Fig.(2) and Table (5) exhibit a maximum of 4 consisted bands very well visible on the gel though tightly associated with relative mobility range from 0.166 to 0. 902 and characterized by a clear inter and intra polymorphism in bands presence and

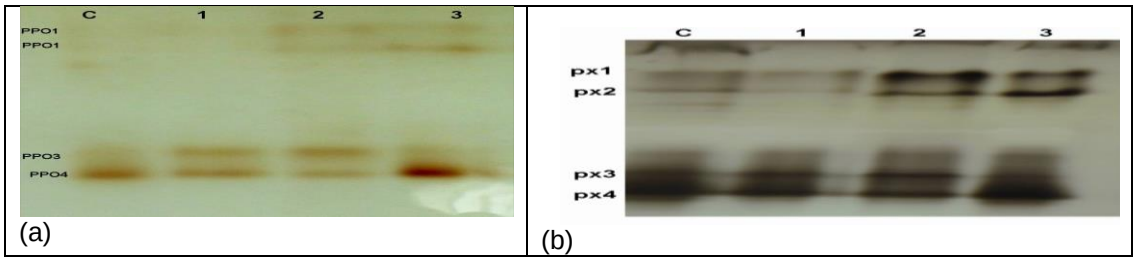

Figure (2) Patterns of isoenzymes (a) polyphenyl oxidase (PPO) isozymes and (b) peroxidase (Px) in transgenic *Populus alba*: line C (untransformed), 1-3: different periods of inoculation (5, 10 and 20 min) with *A. tumeficens*.

absence and variable densities among different Period's inoculation with *A. tumefaciens* and control samples. Shootlets which were infected with *A. tumefaciens* for 20 min and 10 min produced 4 bands with relative mobility (0.166, 0.600, 0.749 and 0.902) and different densities whereas the band at (Rf 0.749) had higher density in inoculation for 20 min while inoculation for 10 min. produced moderate density. The inoculation for 5 min produced two moderate bands at (Rf 0.749 and 0.902), though, control plant produced three bands one of them at (Rf 0.166) with faint density and the others two were moderate density. These results are agreement with that of that Sun et al. (2010).

CONCLUSION

It can be concluded the explants it inoculated with *A. tumeficens* suspension (OD_{600} = 0.8) for 20 min exhibited highly transformation frequency and their expression was highly activity representative in comparison with other different periods of inoculation and the control plant. Analyses supported that there was successful integration of T-DNA into the genome and gene expression of transgenic plants. Results presented here are expected to be helpful for biotechnological studies for poplar improvement.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest".

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AUTHOR CONTRIBUTIONS

MHS, MMAG, MHAH and ASM designed and performed the experiments. MMAG and ASM carried out the experiments, MMAG also wrote the manuscript, MHS and MHAH reviewed the manuscript. All authors read and approved the final version.

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