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Temporal Expression of *Neurospora crassa* Tyrosinase Gene Under The Control of Glucose -Repressible Gene-1(Grg-1) Promoter

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Abstract: In this study a chimeric glucose- repressible gene-1/tyrosinase gene (Grg-1/tyrosinase) was used. This chimeric gene contains 1.558kb fragment of *Neurospora crassa* Grg-1 regulatory region upstream of the tyrosinase coding region. This 1.558 kb fragment of *Neurospora crassa* Grg-1 5' region controls the expression of *Neurospora* tyrosinase in a glucose-repressible manner. The level of the tyrosinase enzyme increases about 5 fold in absence of glucose in the extracellular medium compared to that in the presence of glucose. This highest level is reached within 2 hours after the shift from medium containing glucose to medium without glucose. The level of tyrosinase enzyme returns to the basal level of expression with less than 10 minutes following the shift of fungus mycelia from medium without glucose to medium with glucose. High ratio of the expression of the tyrosinase in absence of glucose to that in presence of glucose is obtained with 3% and 4% glucose concentration in the extracellular media. The glucose repression is obtained with a minimum glucose concentration as much as 1% in the growth media. The level of the expression of the endogenous tyrosinase gene is very low and does not respond to the glucose repression effect. The repression of tyrosinase expression under Grg-1 regulatory region was not obtained with other carbon sources such as arabinose, sorbitol, xylose,glycerol, starch, maltose, ribose, manitol, sucrose, lactose and fructose. In the developmental stages: conidia and mycelia the expression of the tyrosinase gene is repressed by glucose in both stages. The level of the expression of the tyrosinase enzyme in presence of glucose in the conidia is higher than that in the mycelia. However, in the absence of glucose the level is higher in the mycelia than that in conidia.

Key words: *Neurospora crassa,* Glucose repression, Glucose repressible gene, Tyrosinase, chimeric genes, gene regulation

INTRODUCTION

Glucose and related sugars repress the transcription of genes encoding enzymes required for the utilization of alternative carbon source (Gancedo, J.M. 1998). Under the availability of nutrient-sufficient condition, the filamentous fungus *Neurospora crassa* grows through the formation of multinucleate vegetative cells. In condition such as nutrient deprivation, desiccation and light cues, the fungus starts a conidiation developmental program (Kothe *et al.,* 1998; Springer, 1993). The expression of *Neurospora* conidiation program is under the regulation of at least three environmental factors: carbon catabolite repression (glucose repression), the circadian rhythm and the blue light. Glucose-repressible gene-1 (Grg-1) is one of the earliest of *Neurospora crassa* genes expressed during the conidiation process (Loros *et al.,* 1989). (Grg-1) was isolated initially by differential screening of cDNA library prepared from induced and repressed culture of *Neurospora crassa* the cDNA was used to isolate Grg-1 gene which was also sequenced. The initial fragment of DNA containing the Grg-1 gene contains 750 nucleotides upstream of the start of transcription (McNally and Free, 1989). The rest of the 5' region up to 2.3kb was isolated and sequenced (Tarawneh, 1989). The expression of the endogenous grg-1 gene at the level of mRNA transcription is about 50 fold in absence of glucose than in the presence of glucose (McNally and Free, 1989). A chimeric gene of Grg-1 gene containing 500 nucleotides of *E.coli* B-glucorinidase gene in the middle of Grg-1 gene was engineered (Tarawneh, 1989).This chimeric gene codes for 1.2 kb transcript. However, the endogenous grg-1 gene codes for 0.7kb transcript which was used as internal control in the transformation experiment. This grg-1/glucorinidase gene was introduced into *Neurospora* qa-2 arom9 inv albino mutant via pRAL-1 plasmid containing the qa-2 gene. The 750 nucleotides upstream of Grg-1 start of transcription was found to regulate the expression in a glucose repressible manner (Tarawneh, 1989). 5'deletion of the 750 nucleotides fragment demonstrated the presence of a cis -acting element about 17 nucleotides required for the transcription of Grg-1 in glucose regulated manner.

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This gene was also isolated as a clock-controlled gene (ccg-1) (Loros *et al.,* 1989; Dunlap and Loros, 1990). The 750 nucleotides fragment was found to regulate the expression of Grg-1 gene in a clock-controlled way (Liu,Y. and Bell-Pedersen, D., 2006; Tarawneh,1997). Blue light was found to induce the expression of Grg-1 (ccg-1) gene (Arpaia *et al.,* 1995).

The 1.558kb fragment upstream of Grg-1 gene was cloned in front of the tyrosinase gene. This Grg-1/tyrosinase construct was used to transform qa-2 arom9 inv mutant by cotransformation experiment with another plasmid containing the hygromyosin gene as a selectable marker (kothe and Free, 1993). The resulting transformant GTH16 contains the Grg-1/tyrosinase chimeric gene.

Most of the previous works indicate when Grg-1 gene is on and when its repressed, but it is not known how fast the switch takes to shift from the activation state to the repressed state. In this study we investigate the temporal and developmental expression of the tyrosinase gene under the control of 1.558 nucleotides of Grg-1 regulatory sequences in absence and presence of glucose. We also studied the effect of other sources of carbon on the expression of the Grg-1/tyrosinase gene in the absence of glucose.

MATERIALS AND METHODS

Plasmids and Transformation:

EcoRV / XhoI DNA fragment containing the tyrosinase coding region was introduced in B-bluescript plasmid. This plasmid was called pTY103 and contains single sites for EcoRI and XbaI for the introduction of regulatory sequences upstream of the AUG codon of tyrosinase. The tyrosinase sequences contain 18 nucleotides upstream of the translation initiation codon. These 18 nucleotides contain the consensus sequences preceding the translational site AUG of many *Neurospora* genes (Table 1).1.558 kb DNA fragment containing the regulatory region of Grg-1 introduced upstream of the tyrosinase gene (Kothe and Free, 1993). GTH16 *Neurospora crassa* transformant was obtained by cotransformation *Neurospora* mutant qa-2 arom9 inv with the plasmid containing the construct Grg-1/tyrosinase and another plasmid containing the hygromyosin resistance gene as a selectable marker (Kothe and Free, 1993).

Media and Culture Conditions:

Neurospora GTH16 transformant conidia were grown on 2% Vogel, 2% glucose as carbon source (Davis and De Serres, 1970), 2% agar and 5X arom solution. Vogel solution was prepared as 50X stock solution (Na_3 citrate.$5H_2O$ 150g, KH_2PO_4 250g, NH_4NO_3 100g, $MgSO_4$.7 H_2O 10g, $CaCl_2$.2H2O (predissolve in 20 ml H_2O: add slowly) ,Biotin solution 5ml (Biotin solution is prepared by dissolving 5 mg of biotin in 100 ml of 50% (v/v) ethanol and stored at 4°C, Trace element 5ml: (Citric acid.1 H_2O 5 g, $ZnSO_4$.$7H_2O$ 5g, $Fe(NH_4)_2(SO_4)_2$.$6H_2O$ 1g, $CuSO_4$ H_2O 0.25g, $MnSO_4$.H_2O 0.05g, H_3BO_3 0.05g, Na_2MoO_4.$2H_2O$ 0.05g). Arom solution was prepared as 250X solution (1X solution contains 40 microgram/ml of each of the following amino acids L-tyrosine, L-tryptophane, L-phenylalanine, plus 0.25 microgram /ml para-aminobezoic acid (Giles *et al.,* 1967). The conidia were allowed to grow at room temperature for about 2 weeks. They were then harvested using sterile Pasteur pipette and suspended in 10 ml sterile distilled H_2O. The suspended conidia were filtered using sterile glass wool and their number was determined using hemocytometer. The number of conidia inoculated in each culture is fixed to 10^8conidia/ml. The conidia were cultured in a medium containing 2 % Vogel, 2 % glucose and 5X arom solution. The culture is then incubated at 30 ^{0}C and shacked at 150 rpm overnight. The mycelia were then harvested by filtration and transferred to medium without glucose containing 2 % Vogel and 5 X arom solutions. The mycelia were gown for another 2 hours and harvested for the tyrosinase enzyme assay.

Enzyme Assay:

The mycelia were added to 10 ml of staining solution containing 25mg/ml L-tyrosine and 1 % Triton. L-Tyrosine is the substance for the tyrosinase enzyme. It is converted to melanin and the product could be photographed or measured spectrophotometrically at 295 nm which give the maximum absorbance. The Triton allows the lyses of the cell membrane and do not inhibit the function of the enzyme.

Temporal Culture:

The conidia were grown in presence of glucose overnight as described in the growth culture. The mycelia were harvested and shifted to medium without glucose for 5min, 10min, 20min, 30min, 60min and 120min. The cells were allowed to grow for the appropriate time in absence of glucose and then harvested and tested for the tyrosinase activity under the control of Grg-1 gene regulatory region.

Effect of Glucose Concentration:

Conidia were inoculated in a medium containing 2 % fructose (0 % glucose), 1 % glucose, 2% Glucose, 3 % glucose and 4 % glucose. After 12 hours of incubation the mycelia were shifted to medium with the same glucose concentration and to medium without glucose except for the culture grown in presence of fructose the mycelia were shifted to medium without glucose and a medium containing 2 % glucose. The mycelia were allowed to grow for 2 hours and then harvested and tested for the activity of the tyrosinase enzyme.

Developmental Culture:

The conidia were harvested from *Neurospora* culture on 2% Vogel, 2 % glucose. 2 % agar and 5 x arom solutions. The conidia were incubated in a medium with and without glucose and after 2 hours of incubation the tyrosinase activity was tested. For the mycelia culture the conidia were inoculated in a liquid containing 2% Vogel, 2 %glucose, and 5 x arom solution and allowed to grow overnight and the next day the mycelia was shifted to medium without glucose. This culture will prevent the formation of conidia and allow testing the expression of the Grg-1/tyrosinase gene in the mycelia.

Neurospora Wild Type Culture:

The conidia of the *Neurospora crassa* wild type strain 74-23OR-1A were grown in 2% Vogel, 2% glucose overnight at the same condition of temperature and shaking and the next day the mycelia were shifted to a medium with and without glucose for 2 hours and then tested for the activity of the endogenous tyrosinase gene as described in the enzyme assay.

Data Analysis:

The results were expressed as mean ± SD and analyzed statistically by student's t-test employing SPSS program. A P values above 0.05 was considered non significant.

RESULTS AND DISCUSSION

Glucose repression phenomenon (carbon catabolite repression) is a known system of gene regulation in microorganisms (Gancedo, J. M.,1998). The cells grown in a medium containing glucose repress the expression of many genes required for the metabolism of alternate carbon sources such as yeast genes, ADH2 (alcohol dehydrogenase),Gal (galactose utilization) genes,Mal6 (Maltase) and SUC2 (Invertase) (Beir *et al.,* 1985; Sarokin and Carlson,1985; Post-beittenmiller *et al.,* 1984; West *et al.,* 1984; Hong and Marmur,1987, Celenza and Carlson, 1986).

In the filamentous fungi *Neurospora crassa* a number of glucose repressible genes have been studied such as invertase, amylases, an ATP-dependent high- affinity glucose transport system and Grg-1(ccg-1) gene (Lee and Free, 1984; Sigmund *et al.,* 1985; Scarbrough *et al.,* 1970; Neville *et al.,* 1971; McNally and Free, 1989; Tarawneh, 1989).

Grg-1 was isolated by differential screening of cDNA library with a single stranded DNA probes prepared from glucose-sufficient and glucose starved *Neurospora* cultures (McNally and Free,1989). This gene is isolated as glucose repressible gene, clock-controlled gene, and as a blue-light inducible gene (McNally and Free, 1989; Loros *et al.,* 1989; Dunlap and Loros, 1990; Arpaia *et al.,* 1995). This gene is one of the earliest genes expressed in the *Neurospora* conidiation program. We report the use of Grg-1/tyrosinase chimeric gene (kothe and Free, 1993) to study the activity of the tyrosinase gene under the control of 1.558kb regulatory region of grg-1. The restriction map of this construct and the complete sequence of the 1.558kb regulatory region of Grg-1 gene are shown in (Fig. 1 and Fig. 2). The 18 nucleotides upstream of the tyrosinase gene start of translation contain the consensus sequences preceding the translational start sites in many *Neurospora genes* (Table 1).

To study the effect of glucose and the temporal expression of Grg-1/tyrosinase gene under the glucose deprivation or glucose –sufficient conditions, GTH16 transformant strain was used. This transformant derived from RLM5, a strain with an al-2; arom-9; inv; qa-2, which contains multiple copies of Grgr-1/tyrosinase chimeric reporter gene (Kothe and Free, 1993). The tyrosinase catalyzes the only enzymatic step in the biosynthesis of the pigment melanin from L-tyrosine or DOPA.The level of tyrosinase is assayed specrophotometrically at 295nm.It was found that the 1.558 kb regulatory region of grg-1 regulates the tyrosinase in a glucose repressible manner (Fig. 3). The maximum effect was obtained with 3 % and 4 % glucose concentration. This probably due to the availability of the glucose in the extracellular medium. The level of the endogenous tyrosinase in the wild type strain 74-23OR-1A is very low in absence of

Table 1: This table shows the consensus sequences preceding the translational site ATG of some *Neurospora crassa* genes including the 18 nucleotides upstream of the tyrosinase gene used in the chimeric gene Grg-1/ tyrosinase.

Gene	CA rich region	Consensus sequences	ATG	Reference
B-tubulin	ACCAAACC	CAAG	ATG	Orbach *et al.,* 1986
Am	TCACCACC	CAAA	ATG	Kinnaird *et al.,* 1982
H4	CCAACACA	CAAA	ATG	Woudet, *et al.,* 1983
Trp-1	CCAACACA	CACA	ATG	Scechttman, *et al,* 1983
ATPase subunit	ACCCAACC	CAAA	ATG	
Grg-1	ACCCCTCA	CAAA	ATG	McNally, *et al.,* 1989
His3	ACCAAACA	CACA	ATG	Legerton *et al.,* 1985
Crp5	TCAAGACA	CAAG	ATG	Wang, *et al.,* 1993
Ubi: :crp-6	CCTTACCA	AATC	ATG	Tarawneh *et al.,* 1994
Crp-1	CACCACCA	CAAC	ATG	Shi, *et al.,* 1991
Cox5	AGCCGACA	CAAC	ATG	Sachs, *et al.,* 1989
Cyt-21	CCACCACC	CAAC	ATG	Kuiper, *et al.,* 1988
Qa-2	ACCCAACC	CACA	ATG	Alton, *et al.,* 1982
Qa-3	ACCACACA	CACC	ATG	Alton, *et al.,* 1982
Qa-4	CAGACCCA	CGCC	ATG	Rudlege, 1984
Cys-3	GCAAAACA	CACA	ATG	Fu, *et al.,* 1989
CuMT	AACAACCA	CAAA	ATG	Munger, *et al.,* 1985
Cpc-1	CCAAAACA	CAAG	ATG	Paluh *et al.,* 1988
H3	AACACACA	CACA	ATG	Woudet, *et al.,*1983
Con10	AAACAACA	CAAC	ATG	Roberts, *et al.,* 1988
Qa-1s	CCACCACC	CATA	ATG	Huiet, *et al.,* 1986
ADP/ATP carrier	TTACAGCA	CACA	ATG	Arends, *et al.* 1984
Nrc-1	AACAACTC	CAGC	ATG	Kothe, *et al.,* 1998
Nrc-2	TCACATCA	CAAG	ATG	Kothe, *et al.,* 1998
Tyr	CACATCAA	CAAA	ATG	Kothe, *et al.,* 1993

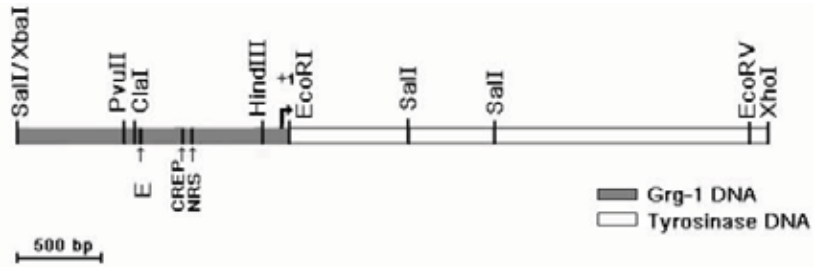

Fig. 1: This figure shows the restriction map of the Grg-1/tyrosinase construct in the GHT16 transformant. The EcoRI/XhoI fragment containing the tyrosinase was first introduced in the B-bluescript plasmid to give the pTY103 plasmid and the 1.558kb Grg-1 regulatory region was introduced upstream of the tyrosinase coding region(Kothe and Free, 1993). The tyrosinase sequences contains 18 nucleotides upstream of the AUG codon. +1 indicates the start of transcription of Grg-1 gene and there are 67 nucleotides of grg-1 sequences downstream of the start of transcription. There are 18 nucleotide of the tyrosinase sequences upstream of the AUG codon. E, CREP and NRS represent the enhancer (ACTCAGTTCGTT), the cyclic responsive like element (GTGCGTCAC) and the *Neurospora* repressor element (TTGCTAGCAA) respectively. The restriction enzyme sites are shown on the map.

glucose and does not respond to the glucose repression system.T-test analysis of the activity of the tyrosinase in the wild type strain 74-OR-1A in presence and absence of glucose showed no significant differences ($P>0.05$). These results indicate that the 1.558 kb grg-1 regulatory sequence contains all the information required for the regulation of tyrosinase in a glucose repressible manner. Fig. 3 shows that the level of induction upon glucose starvation ranges from 2-5 folds compared to the 50 folds of the endogenous Grg-1 activity (McNally and Free, 1989). The level of the tyrosinase was not as high as expected since the transformant contains multiple copies of the Grg1-/tyrosinase gene (Fig. 3). However, t-test statistics showed significant differences between the mean absorbance at 295nm in absence of glucose and in presence of glucose ($P< 0.05$). This low level of expression was also noticed in all *Neurospora* transformants containing other *Neurospora* genes (Kinsey and Rambosek, 1984; Tarawneh, 1989). Despite the low level of the tyrosinase, the Grg-1/tyrosinase chimeric gene was regulated by glucose expression (Fig. 3). The temporal expression of the Grg-1/tyrosinase showed that the gene is induced within 5 minutes after the shift to medium without glucose. Fig. 4 shows that the activity is increased gradually with time and the maximum activity is

	CT	CGAGCCAGGC	TCGCAGGCAC	AAAGCACGCT	-1531
GGGAAGAGGA	GCAACGTTGA	GTGGGCGGGC	CTCGAACCGT	CGGTAGTTCC	-1481
TCTTGCCAAT	GGGATTCGCT	GCCCGTGTTT	GGCGAGCTTG	GTCTCTAGTG	-1431
CAGAGCAACC	CCTCATTTGG	GGAATCCAGC	GAGCAATCTG	TTGCACGTTG	-1381
GGAAAGGCAG	GAGCGCAATC	TTCCTATCAG	CTGCTCACAC	TCGTCAGTGC	-1331
ATCATCGAAG	AGCCGAATTG	CCATGGAGAA	ACAGGATGGG	AATCTGGCAA	-1281
TCCGAGGAAT	CTCGCCACTG	GCTCCAACAG	ATCATTCAAC	CGAGGCAGGA	-1231
GGATCTACCA	ATGCGAGGCG	CCTTTCGTAC	GTTCATTTAC	AAAATTGAAC	-1181
CAAGATCAAG	ATGACCGTGT	TCTCGGTTTG	ATACTATGGA	GCCCACGGCT	-1131
GTTCAAGTCC	TGTGTGAATG	CAAGTCGGCC	GCAGCGGCTC	CTCCCCAGAT	-1081
TTCAGATTGT	AGTATGTACC	TAGCAGTGCG	GGCATAAGTA	GCTAGCTGCA	-1031
CATCAATAGT	GCGGGCTCAG	CTAATGGGAA	TCGTTCTTTG	ACGGCCAGCC	-981
CTGCCATGCG	TCACTGGCCG	GGATAATGGC	CCATGATGCC	TCTTTCCATT	-931
TCCGGAAGTT	TGTGTACTTT	CGCCTAAACA	CGAGATGTGA	TTCTCAGGCA	-881
ACGACGCGGA	AGGAGAGAGC	CCAAAGTCGG	GCGCGTCTTG	TTCCAGCTGT	-831
AGAAGGAGCA	GTCCATCTGC	GTGAATCACG	AGAGAATCAG	CTACTTTGAA	-781
TCGATGGATG	CAGCTACCAG	AAGTGACTCA	GTTCGTTCAA	AGCCACATCA	-731
CTGGGCACTT	CCATTGGGAC	AGGCATTGAT	CGGACGAGAC	CGACTTCTGG	-681
CCGCTTTCAA	CAGCCACATT	ATATCCATGT	CACGGCTACG	CGCGGCCTTC	-631
GGTAACAGAA	AAGCACACAG	ACAGCGATTG	TGACATGGAT	TCGGGCAAAC	-581
GATTGGTGGT	CGCACCAGGT	CACCTGAGTG	TGCAGTGGCT	GCTATTCAGA	-531
TTTCATCTAA	CTGCGGGAGA	GGGGTTCAAA	GGGGCGTGAC	GTCACAGACA	-481
ACGGGTGAAG	GACGAAGATT	GCCTCACTTC	TTTGCTAGCA	ATTTGCCTGC	-431
AAAGAAGGCG	CACATGTCAA	GCAAACAACT	GGGAAACCAC	TATTGAATAC	-381
CCACAATGCA	AAGCTCGGAA	GGTACGTCTT	GATTGCAGTG	TGTCGAGTGT	-331
CAAAAAAGAA	GCAAGTGTTC	ATGCAAGCCA	AAATTGGCAC	CTCCTCCACT	-281
TCTCCGAGTG	CCCCACCCGA	ACCTCCAGGC	GAGATGGCCG	GAACATACCA	-231
TCCGCGTTGG	GATTATGACG	TATCTCCTTC	TTCTTCACAT	GATTCCATCC	-181
CGTTGTTGCT	TGTTTGCGAG	CTGTGACGGG	AGATCGTAGA	TGCCACTTAG	-131
GGCCAGGCAG	GCAGTGCAGG	CAGCCAGGAA	CACAAGCTTC	CAACTTGGTC	-81
ATCTCGATTG	CCGATTAAGG	GAACCAAATG	CCTATATAAG	ACTGTCCTCC	-31
CACCTCCCCA	ATACCATTCT	TTTCTTCTTC	CATCATCAGC	CAACAAAGCA	+20
ATCACATCTT	CACTACTTCA	AATCAACACA	ACACTCAAAC	CACTTTCACA	

Fig. 2: This figure shows the sequences of the 5' regulatory region of Grg-1 gene upstream of the tyrosinase gene. The sequence from Grg-1 start of transcription to -750 nucleotide was sequenced by McNally and Free, 1989 and the sequences from-750 to -1558 nucleotides was sequenced by Tarawneh. 1989. The enhancer (ACTCAGTTCGTT), the cyclic responsive like element (GTGCGTCAC) and the *Neurospora* repressor element (TTGCTAGCAA) are indicated by squares in the sequence shown in (Fig 2). The starts of transcription are indicated by arrows. The start of translation ATG is underlined.

obtained after 2 hours from the shift to glucose -insufficient Vogel medium. It was found that upon the shift from mycelia grown in the absence of glucose for 2 hours to medium with glucose, the tyrosinase activity is reduced to the background level within 10 minutes and stay in this state for the following times 20, 30, 60 and 120 min after the shift. This results indicated that the gene switch from the induction to the repression and vice versa within the first 5 minutes of the switch shift (Fig. 3). This rapid response of the Grg-1 gene demonstrated that the metabolism of the fungus involved this gene adjust itself to the environmental cues rapidly and that the switch off is more sensitive to the presence of glucose in extracellular medium.. This is

not surprising in the light that Grg-1 regulatory region respond to three factors: glucose deprivation, circadian rhythm and blue-light induction. It is also known that Grg-1 is involved in the conidiation program that responds to carbon source limitation and desiccation. We are interested in how all these factors interact and manage to regulate the Grg-1 expression in a subtle way.

Different sources of carbon were tested to repress the induced Grg-1/tyrosinase gene (Fig. 5).The conidia were first grown overnight in presence of glucose then shifted to medium without glucose for 2 hours then shifted to medium containing the appropriate source of carbon: arabinose, sobitol, xylose, glycerol, starch,

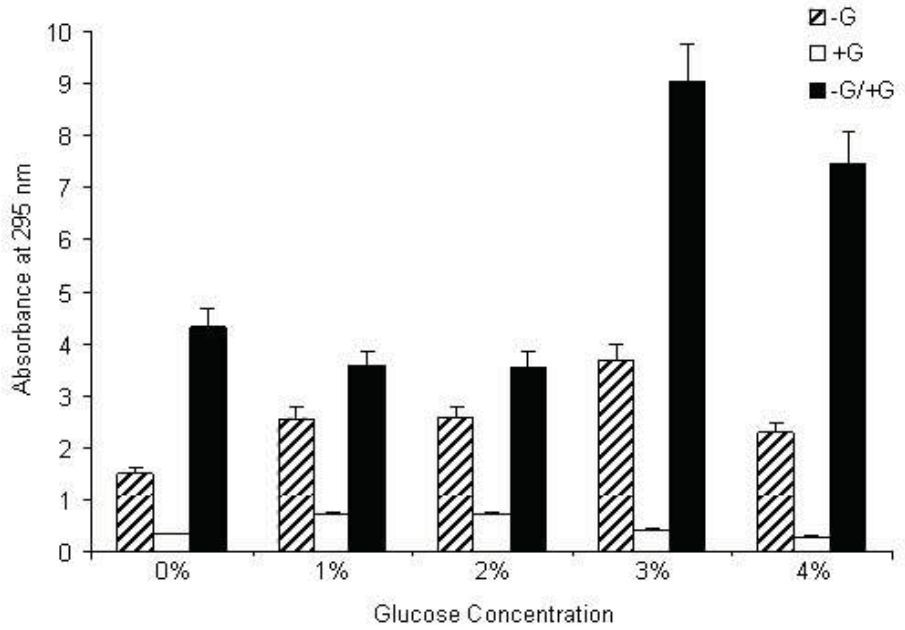

Fig. 3: This figure shows the effect of glucose concentration on Grg-1/tyrosinase expression. The conidia were grown in 2 %fructose(0 % glucose), 1% glucose, 2% glucose, 3% glucose and 4 % glucose overnight and then the mycelia was shifted to medium with the same concentration of glucose and without glucose for 2 hours except the culture in presence of fructose the mycelia was shifted to medium without glucose and a medium with 2% glucose. The absorbance was measured at 295nm after staining the mycelia obtained from each culture in presence (+G) and in absence of glucose (-G). The ratio of the absorbance (-G/+G) is shown in the figure. The absorbance at 295nm is shown on the Y axis and the glucose concentration is shown on the X axis.

maltose, ribose, manitol, sucrose, lactose and fructose. All of the carbon sources used fails to repress the Grg-1/tyrosinase gene (Fig. 5). It was found that even the carbon source that produce glucose upon degradation fail also to repress the Grg-1/tyrosinase gene and that this phenomenon is glucose specific. The level of the endogenous tyrosinase gene activity in the wild type *Neurospora crassa* strain 74-23OR-1A does not respond to the glucose repression system (Fig. 5). GTH16 mycelia grown in presence and absence of glucose were used as negative and positive control in studying the effect of carbon sources on Grg-1/tyrosinase expression (Fig. 5).

To study the developmental expression of Grg-1/tyrosinase gene, the activity of the tyrosinase was followed in conidia and mycelia in absence and presence of glucose (Fig. 6). The Grg-1 gene was found expressed in both conidia and mycelia. The level of the tyrosinase was significantly high in conidia in presence of glucose compared to that in mycelia ($P< 0.05$), however, the level of the tyrosinase in conidia is significantly lower than that in mycelia without glucose in the extracellular medium ($P< 0.05$) (Fig. 6).This result is not surprising since Grg-1 is one of the earliest expressed genes in *Neurospora* conidiation program (Loros *et al,* 1989). It is also known that desiccation or carbon deprivation induces the asexual sporulation process of conidiation (Ricci *et al.,* 1991). This result also indicated that Grg-1 gene is expressed and regulated by glucose repression mechanism.

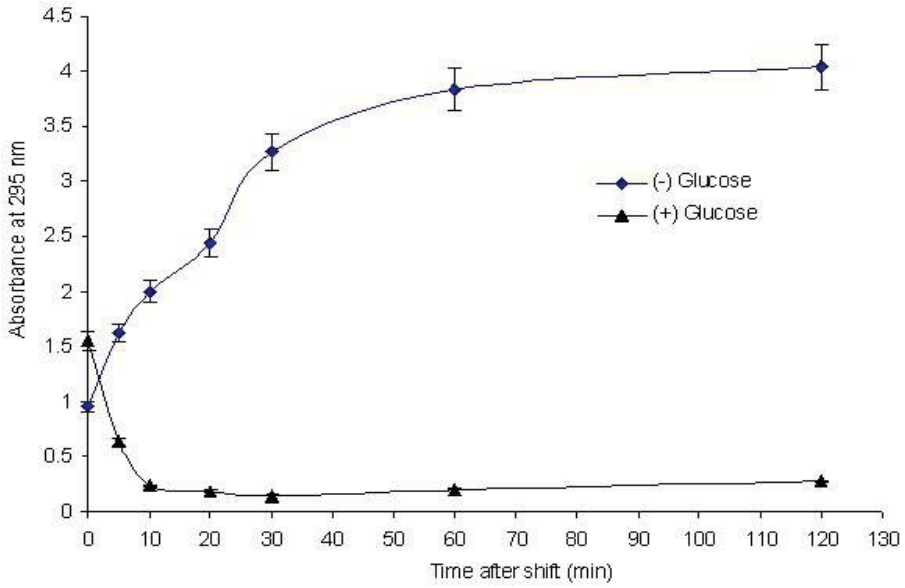

Fig. 4: This figure shows the temporal expression of the Grg-1/tyrosinase gene in presence and in absence of glucose. 10^8 conidia were inoculated in Vogel medium as described in the materials and methods and grown overnight at 30 °C with shaking at 150 rpm. The next day the mycelia were shifted to medium without glucose for 5, 10, 20, 30, 60, 120 min. The mycelia were then stained for the activity of the tyrosinase enzyme under the control of the regulatory region of Grg-1 gene. To study the temporal repression, the conidia were grown first in presence of glucose overnight then the next day the mycelia was shifted to medium without glucose to induce the expression for about 2 hours then the mycelia was harvested and shifted to medium with glucose for the appropriate time and then stained for the activity of the Grg-1/tyrosinase construct.

This figure shows the absorbance at 295nm of each stained culture at the appropriate time after the shift to medium without glucose or with glucose. The absorbance is shown on the Y axis and the time in minutes is shown on the X axis. The conditions of the culture were as described in the materials and methods.

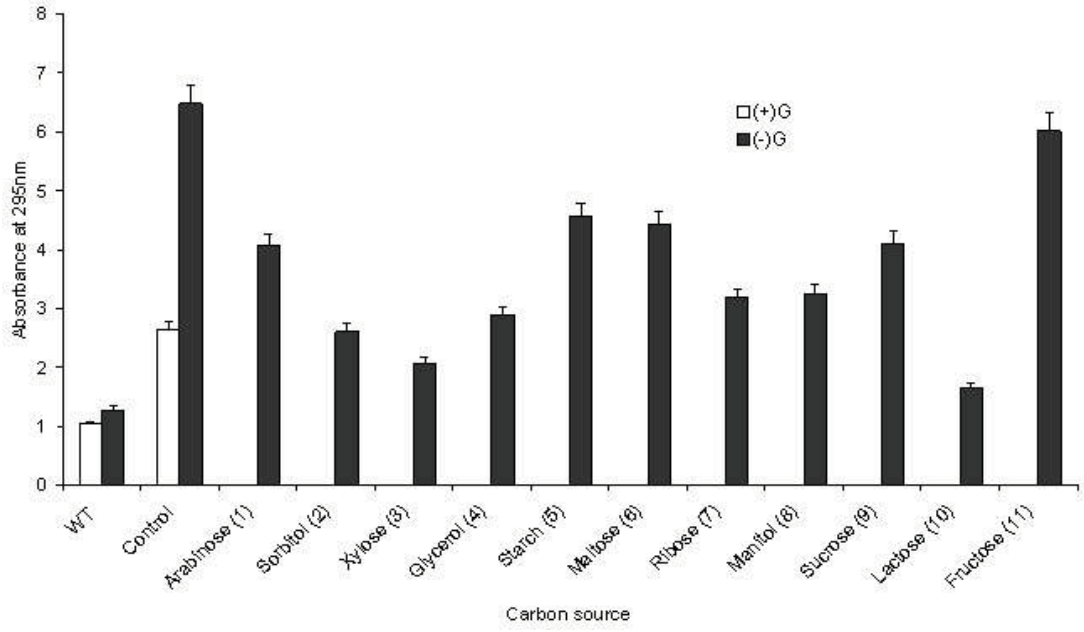

Fig. 5: This figure shows the effect of different carbon sources on the expression of Grg-1/tyrosinase gene. GTH16 transformant conidia were inoculated in 2% glucose, 2 %Vogel and 5 X arom solutions. The conidia were allowed to grow overnight at 30°C with shaking at 150 rpm. The next day the mycelia was harvested and shifted to medium containing 2% of the following source of carbon: 1-arabinose, 2-sorbitol, 3-xylose, 4-glycerol, 5- starch, 6- maltose, 7-ribose, 8-manitol, 9-sucrose, 10-lactose and 11-fructose. This figure shows also the expression of the endogenous tyrosinase gene in the wild type *Neurospora crassa* strain 74-23OR-1A and a control showing the expression of the Grg-1 /tyrosinase in absence and presence of glucose.This figure shows the absorbance at 295nm for each stained culture after the shift to medium without glucose and with the appropriate source of carbon as indicated on the X axis. The absorbance at 295 nm is shown on the Y axis.

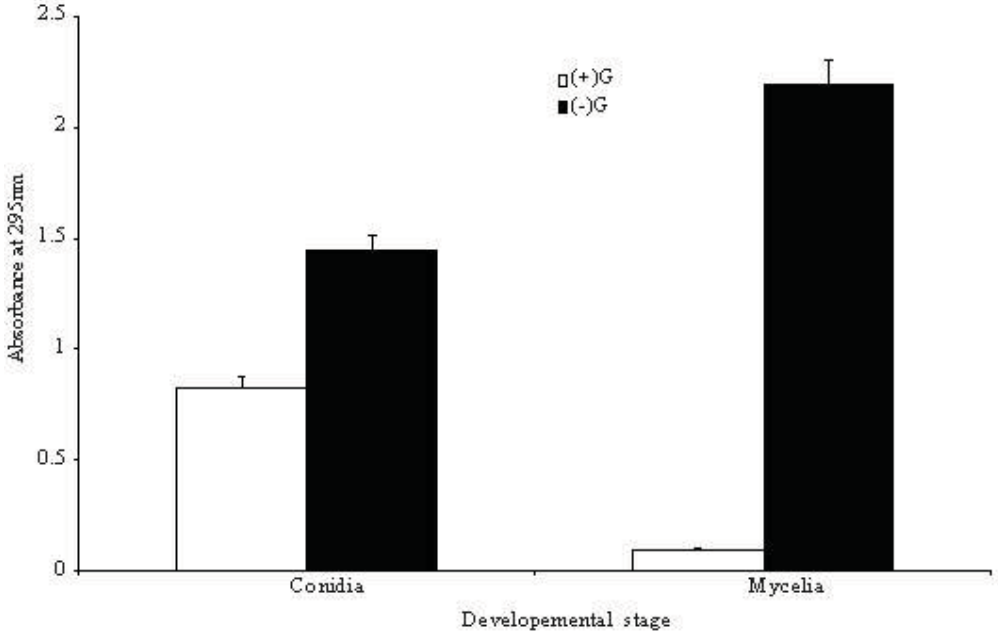

Fig. 6: This figure shows the expression of the grg-1/tyrosinase gene in the developmental stages; conidia and mycelia. 10^{8} Conidia were inoculated in 2 %Vogel and 5X arom medium with and without glucose. The two cultures were allowed to grow at 30 C for two hours and with shaking at 150 rpm.The conidia were harvested separately by centrifugation and stained for the expression of grg-1/tyrosinase. To study the expression of the Grg-1/tyrosinase gene in mycelia, conidia were inoculated in 2%Vogel, 2 % glucose and 5 X arom solutions. The conidia were grown overnight at 30C with shaking at 150rpm. The next day the mycelia were harvested and shifted to medium without glucose and with glucose and allowed to grow for 2 hours .The mycelia were then stained for the expression of the Grg-1/tyrosinase gene. The figure shows the absorbance at 295 nm for each developmental stage conidia and mycelia.

The results reported herein show that Grg-1/tyrosinase is regulated in a glucose repressible manner and the switch from the induction state to the repressed state is obtained within 5 minutes after the shift from medium without glucose to medium with glucose and vice versa .The results also indicated that the phenomenon is glucose specific since none of the alternate carbon sources fail to repress the induced Grg-1/tyrosinase gene. The level of Grg-1 expression was found higher in the conidia in the repressed state compared to that in the mycelia; however the level of the expression is higher in the mycelia than conidia in the induced state. This is due to the involvement of Grg-1 in the sporulation process of conidiatian.

Further research is required to explore the relation between the different factors affecting the expression of Grg-1 gene and the significance of the triple regulation of this gene with glucose, circadian rhythm and blue light.

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