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Effect of Quercetin and Epicatechin on the Transcript Expression and Activity of Antioxidant Enzymes in Tobacco Seedlings

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ABSTRACT

The aim of this study was to see the effect of flavonoids quercetin and epicatechin on the transcript expression and activity of antioxidant enzymes. For this, tobacco seedlings were exposed to 50 and 100 µM quercetin and epicatechin for 21 days. The transcript expression level of various antioxidant enzymes like Glutathione-S-Transferase (GST), Glutathione Peroxidase (GPx), Ascorbate Peroxidase (APx), Glutathione Reductase (GR), Catalase (CAT) and Superoxide dismutase (SOD) were monitored in the root and shoot of exposed seedlings through reverse transcriptase PCR. The activity of these enzymes was studied spectrophotometrically. Interestingly, 50 µM epicatechin and 100 µM quercetin exposures were found to increase the expression of genes encoding antioxidant enzymes in shoot. In tobacco root, only Glutathione-S-Transferase (GST) and GPx expression was increased with 50 µM epicatechin and 100 µM quercetin exposures. Activity assay of all the enzymes showed similar trend to that of the transcript expression in shoot tissue. While in root, except CAT and SOD other enzymes activity also showed similar trend to that of expression pattern. Results have suggested the possible regulation of antioxidant enzymes by these two flavonoids at transcriptional and post-transcriptional level. Additionally, appropriate levels of such flavonoids seem to be essential for such regulations.

Key words: Antioxidant enzymes, epicatechin, quercetin, tobacco, transcript expression

INTRODUCTION

Antioxidant properties of flavonoids make them to involve in large number of plant functions (Koes *et al*., 1994; Olajuyigbe and Afolayan, 2011). Flavonoids have been documented for providing the protection against oxidative damage, age related diseases, coronary heart diseases and certain cancers by acting as a free radical scavengers (Ross and Kasum, 2002; Lee *et al*., 2003; Alipoor and Rad, 2012). It has been shown through *in vitro* studies that flavonoids like quercetin and epicatechin gallate have about five fold higher total antioxidant activities than vitamins C and E (Rice-Evans *et al*., 1996; Rice-Evans *et al*., 1997). For this reason, there is a need for the development of important food crops having optimum level of these flavonoids.

This free radical scavenging property of flavonoids helps the plants to protect themselves against various stresses. Reactive oxygen species (ROS) such as superoxides, H_2O_2 and hydroxyl molecules are generated in plants on being exposed to different stresses. Generated ROS cause damage to the cell through chain reactions (Rao *et al*., 1996; Jayakumar *et al*., 2007). But the plants protect themselves from harmful effect of such stresses by activating certain defense

mechanisms. Plants detoxify these ROS mainly by two ways, either directly through the action of reduced ascorbate (ASH), glutathione (GSH), carotenoids and tocopherols or through antioxidant enzymes like glutathione-S-transferase (GST), glutathione peroxidase (GPx), ascorbate peroxidase (APx), glutathione reductase (GR), catalase (CAT) and superoxide dismutase (SOD) etc. (Amirjani, 2010; Joseph and Jini, 2010).

Flavonoids have been well characterized *in vitro* and *in vivo* for their antioxidant activity (Cruz *et al*., 1998; Schroeter *et al*., 2006). Many earlier reports have emphasized on antioxidant potential of flavonoids through mechanism of direct free radical scavenging in animals (Hanasaki *et al*., 1994). However, the importance of antioxidant property for flavonoids in plants is still a topic of discussion (Hernandez *et al*., 2009). Different localization of flavonoids also affects their functions. The flavonoids localized in vacuoles are known for their photoprotective, light-screening and pigmentation functions but not for their antioxidative functions (Dixon and Pasinetti, 2010). Therefore, the effect of flavonoids was studied on the expression of genes encoding antioxidant enzymes in plants to see relation of these two pathways.

MATERIALS AND METHODS

Plant material: For germination, tobacco seeds (*Nicotiana tabacum* var. Xanthi) were treated with 10% Tween-20 for 5 min and then with 70% ethanol for 30 sec. Thereafter, seeds were surface sterilized with 0.001% mercuric chloride for 3 min and washed thrice with sterile distilled water. Seeds were germinated on Murashige and Skoog (MS) medium (Sigma) in petri dishes at 25±2°C for 7 days, until cotyledons had emerged and roots reached the length of 1-1.5 cm. Seedlings at this stage were transferred to the plates containing either 50 or 100 μM quercetin and epicatechin (Sigma). The stock solution of 10 mM quercetin and epicatechin were prepared by dissolving in 50% dimethyl sulfoxide (DMSO). Finally, MS medium contained 50 or 100 μM quercetin and epicatechin with 1% DMSO. For controls, seedlings were transferred to MS medium containing 1% DMSO alone. The pH of the medium was adjusted to 5.8 with NaOH/HCl. Seedlings were allowed to grow for the next 21 days. The experiment was conducted under controlled conditions of 25±2°C and 50-60% relative humidity. Thereafter, seedlings were carefully removed from the plates and root and shoot were separated and frozen in liquid nitrogen for further use. To see the relationship between two path ways, tobacco seedlings were exposed with 50 and 100 μM quercetin and epicatechin and thus the transcript expression level and the activity analysis of various antioxidant enzymes was monitored in their root and shoot.

Transcript expression analysis: Total RNA was isolated from 100 mg of treated and untreated tissues by using RNAeasy mini kit (Qiagen). cDNA was synthesized using 1 μg of RNA in the presence of 200 U reverse transcriptase SuperscriptTM III (Invitrogen, USA), 1 μL of 10 mM dNTPs and 250 ng oligo (dT)12-18. Resulting cDNA was used to carry out the PCR reactions with gene specific primers encoding for GR (Forward 5'-CATTGCCAATAAAAATGCCGAGT-3' and Reverse 5'-ATGATATGAGAGAAACCTTCAAC-3'), GST (Forward 5'-GTTTGTCCCTGTTGATATGGCCT-3' and Reverse 5'-CACAGCAGCATCATCTGTGGTC-3'), APx (Forward 5'-GAAGCTTAAGATTTGAA GTTGAA-3' and Reverse 5'-CTTAAAGTAGGAATTGTCAAAC-3'), GPx (Forward 5'-GAAATTTTA GCATTTCCTTGT-3' and Reverse 5'-ACGTGGTGAAATGTTCAAX(GA)AAX(CT)-3'), CAT (Forward 5'-CTGGCCTGAGGATATCTTGCC-3' and Reverse 5'-GACGACAAGGATCAAACCTTGA-3'), SOD (Forward 5'-GTCACGGGACCACATTACAAT-3' and Reverse 5'-CCACAAGCAACCCTTCCACC-3'). These genes encoded enzymes as APx, GR and Cu/Zn SOD are present in chloroplast; GST is in

mesophyll protoplast while GPx and catalase are in peroxisomes. The various gene specific primers used for gene expression were analyzed for linearity between the amount of input RNA and the final RT-PCR products was verified and confirmed. After standardizing the optimal amplification at exponential phase, PCR was carried out under the conditions of 94°C-4 min, 94°C-30 sec, 50-58°C-40 sec, 72°C-1 min for 25 cycles and fractionated on agarose gel electrophoresis and visualized with ethidium bromide staining. The 26S rRNA-based gene primers were used as internal control for expression studies (Singh *et al.*, 2004). The intensity of bands was analyzed densitometrically and presented in the form of bar diagram.

Antioxidant activity assay: Activity analysis of different antioxidant enzymes was also conducted in tobacco shoot and root. Ascorbate peroxidase (APx) activity was assayed following the oxidation of ascorbate to dehydroascorbate at 265 nm ($e = 13.7\ mM^{-1}\ cm^{-1}$) by the modified method of Nakano and Asada (Nakano and Asada, 1981). GST and GR activities were determined by the standard methods described earlier (Habig *et al.*, 1974; Carlberg and Mannervik, 1985). GPx activity was also followed by decrease in A_{340}, resulting from NADPH oxidation (Griger *et al.*, 1993). CAT activity was measured following the standard method (Dhindsa *et al.*, 1981). SOD activity was determined by Nitro Blue Tetrazolium (NBT) photochemical assay method (Beyer and Fridovich, 1987).

Statistical analysis: All the measurements were made in triplicate and all values are represented as Mean±SD. The $p<0.05$ was considered significant.

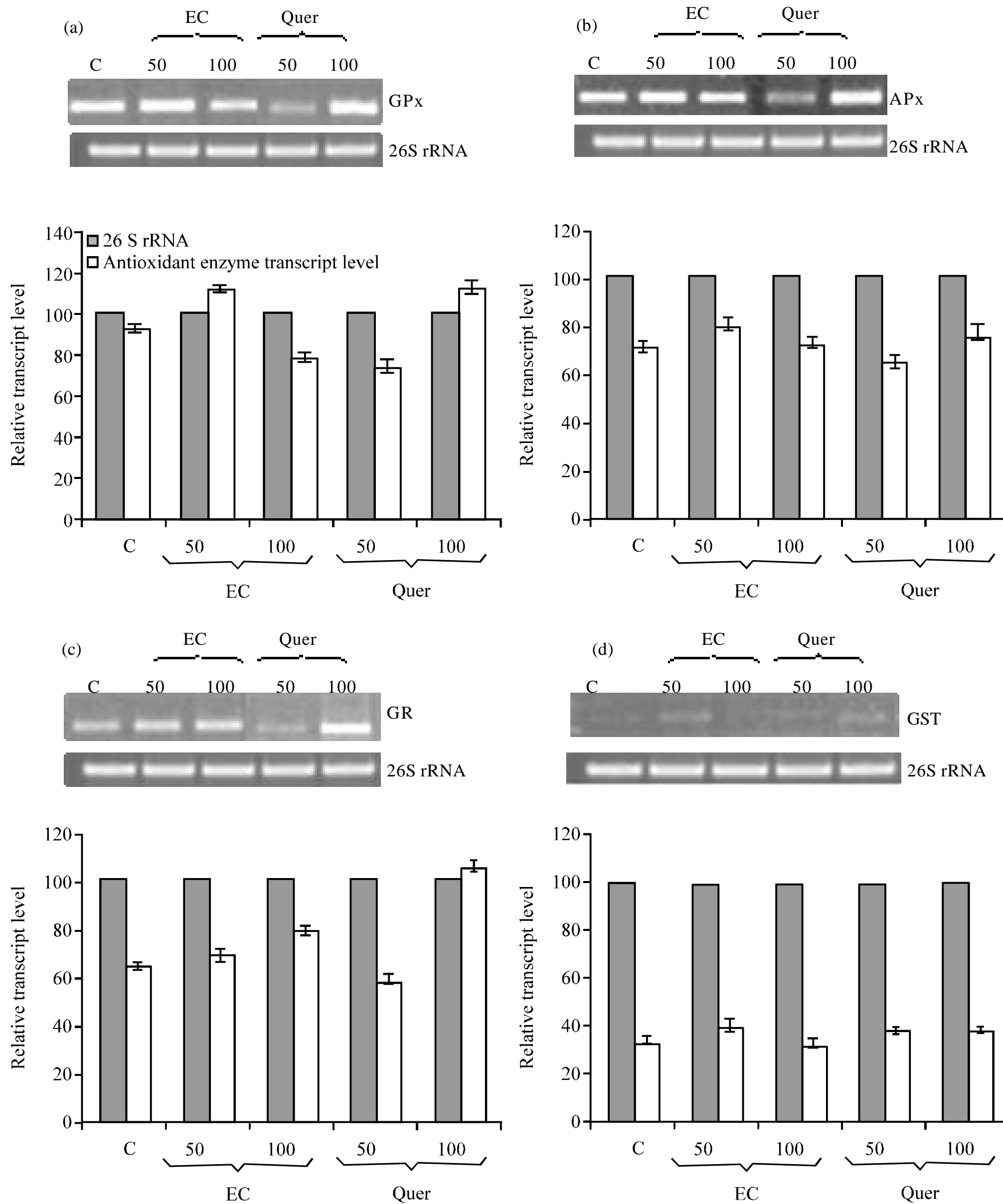
RESULTS AND DISCUSSION

In the present study, influence of exogenous application of quercetin and epicatechin was studied on the transcript level and enzymatic activities of six antioxidant enzymes i.e., Glutathione S-Transferase (GST), Glutathione peroxidase (GPx), Ascorbate peroxidase (APx), Glutathione reductase (GR), Catalase (CAT) and Superoxide dismutase (SOD) in tobacco shoot and root. In tobacco shoot, 50 µM epicatechin exposures enhanced the expression of all the six enzymes GST, GPx, APx, GR, CAT and SOD. In contrast to other enzymes, GR expression was still increased with 100 µM epicatechin. The 50 µM quercetin either decreased or has no effect on the expression of genes encoding antioxidant enzymes in tobacco shoot. However, application of 100 µM quercetin increased the expression of all six genes encoding antioxidant enzymes (Fig. 1). Results suggest that these two flavonoids have effected transcript expression of genes encoding antioxidant enzymes. However, they were effective at different concentrations. Epicatechin was effective at lower concentration while quercetin was at higher concentration. It has been shown earlier that flavonoids are effective in preventing oxidative stress caused by ROS and thus reduce cell damage by activating different antioxidant enzymes (Sati *et al.*, 2010). Similar increased expression of flavonoid biosynthetic pathway genes have been earlier reported in tobacco shoots exposed to lower dose of epicatechin and quercetin (Mahajan *et al.*, 2011).

In tobacco root, GR expression was not affected upon application of either of flavonoids. The APx expression was not affected by epicatechin. But the expression of APx was decreased upon quercetin application. While GST and GPx expression was increased in tobacco root with 50 µM epicatechin and 100 µM quercetin exposures, whereas CAT and SOD expression showed reverse behaviour (Fig. 2). Earlier literature has suggested differential level of expression of genes encoding antioxidant enzymes in various parts of the plant (Mohanpuria *et al.*, 2007;

Gill and Tuteja, 2010). Present results support this and further documented that this differential expression seem to be under the regulation of flavonoids. Results also documented that expression is dependent on the concentration and nature of flavonoids. Similar increase in activity of antioxidant enzymes like SOD, CAT, GPx have been reported in *Vigna unguiculata* roots and shoots exposed to different concentrations of nickel (Eriyamremu and Lolodi, 2010).

To check whether transcriptional or post-transcriptional regulation, activity analysis of all these enzymes was conducted in tobacco shoot and root tissues exposed to epicatechin and quercetin. In tobacco shoot, activity of all the enzymes showed similar trend to that of the transcript expression (Table 1). Significant increase in activity of all the six enzymes GST, GPx, APx, GR, CAT and SOD was reported with 50 μM epicatechin exposures. Whereas, 50 μM quercetin led to the increase in



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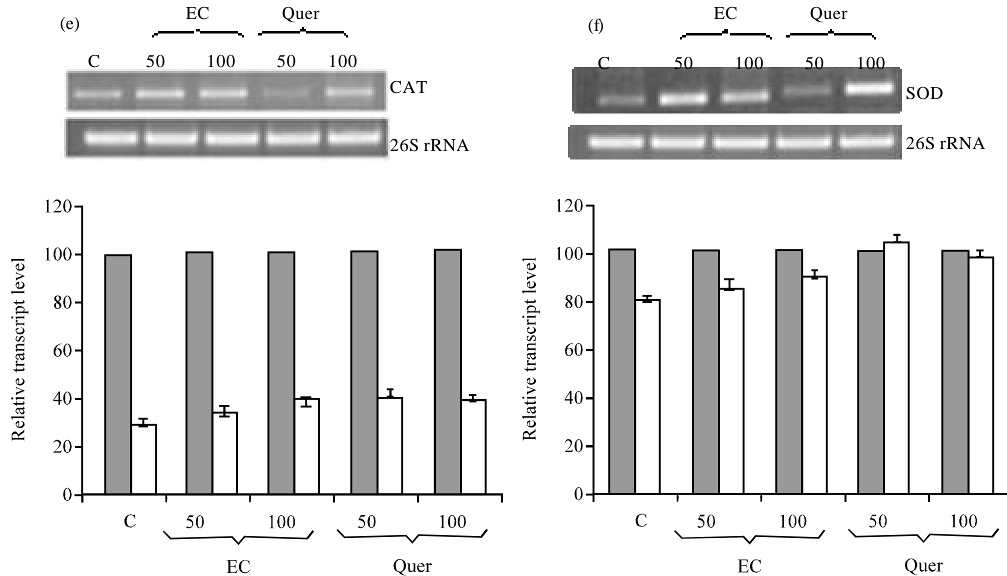


Fig. 1(a-f): Changes in the transcript level of (a) Glutathione peroxidase (GPx), (b) Ascorbate peroxidase (APx), (c) Glutathione reductase (GR), (d) Glutathione S-transferase, (e) Catalase (CAT) and (f) Superoxide dismutase (SOD) in tobacco shoots in response to epicatechin (EC; 50 and 100 μM) and quercetin (Quer; 50 and 100 μM) different treatments. Bar diagram shows relative transcript levels of the respective amplified bands. Expression analysis was repeated at least three times and representative one time gel pictures are presented. Data are means of three measurements±SD. Black and grey bars showed 26S rRNA and antioxidant enzyme transcript levels, respectively. C: Control, EC: Epicatechin, Quer: Quercetin.

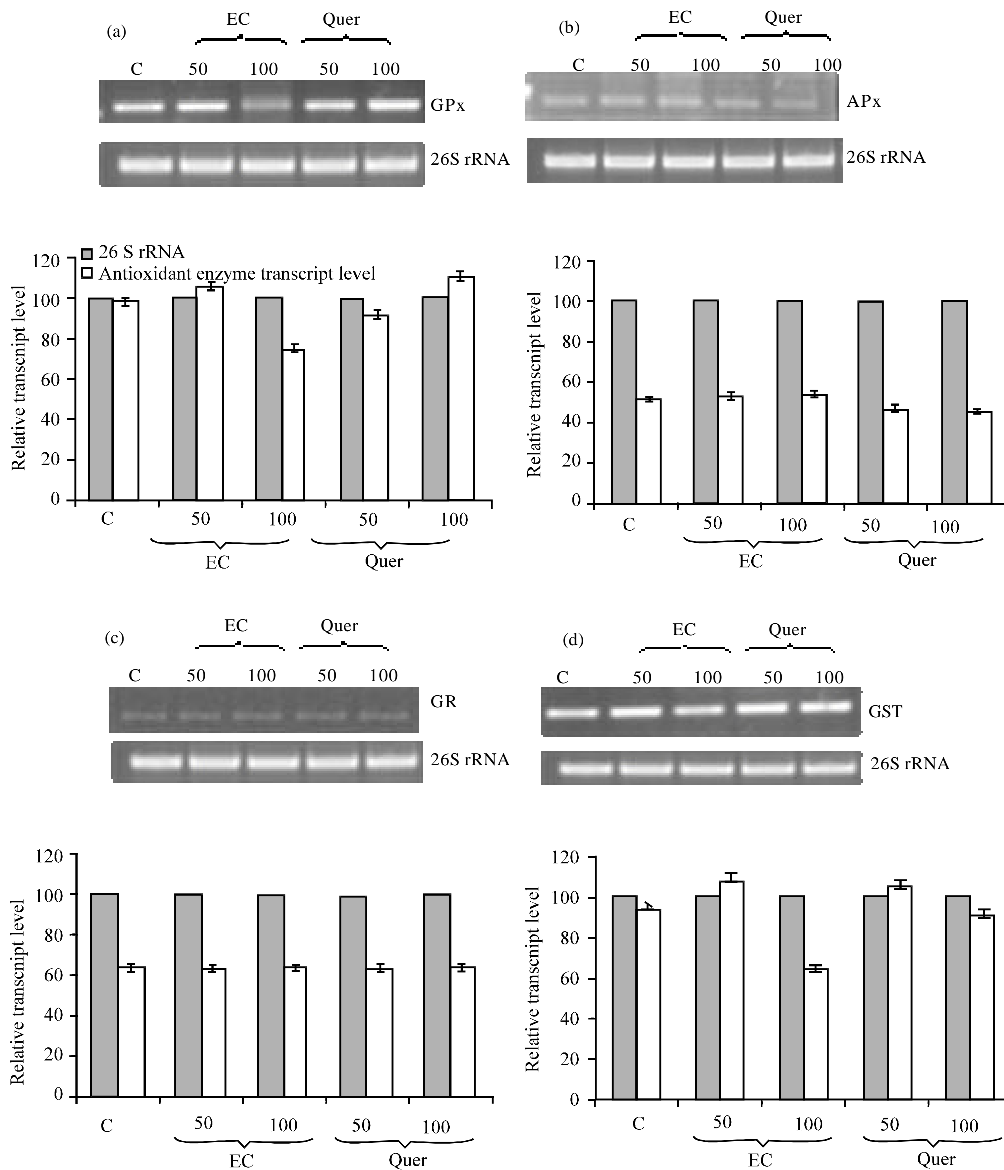
Table 1: Antioxidant enzyme activity in tobacco shoots and roots in response to epicatechin and quercetin treatments

		Epicatechin (μM)		Quercetin (μM)	
Samples	Control	50	100	50	100
Shoot					
GPx	967.0±10.8[a]	1680±50.24[b]	940±58.2[a]	884±34.4[a]	1760±62.8[b]
APx	54.2±1.2[a]	88.6±1.4[b]	62.9±1.8[a]	89.0±1.2[b]	58.0±1.3[d]
GR	10.3±0.4[a]	12.9±0.7[b]	10.96±0.6[a]	11.0±0.7[a]	25.4±0.9[c]
GST	31.7±0.9[a]	50.2±1.0[b]	34.9±0.8[a]	64.8±1.3[c]	60.0±1.2[c]
CAT	17.0±0.8[a]	22±1.2[b]	8.8±0.2[c]	15.2±0.5[a]	25±1.0[d]
SOD	5.8±0.2[a]	13.5±0.9[b]	8.5±0.1[c]	15.8±0.6[b]	21.7±0.9[d]
Root					
GPx	325±21.2[a]	373±22.5[a]	273±14.8[b]	305±20.7[a]	334±25.8[a]
APx	137±2.5[a]	152.1±4.8[b]	162.8±6.3[c]	125.1± 5.9[a]	110.4±5.2[d]
GR	10.96±0.6[a]	9.16±0.4[b]	11.6±0.6[a]	15.0±0.8[c]	13.8±0.9[c]
GST	14.58±0.9[a]	24.2±1.0[b]	9.99±0.4[c]	30.4±0.7[b]	12.8±0.8[c]
CAT	9.2±0.2[a]	11.3±0.4[b]	13.4±0.7[c]	20.5±1.2[d]	15.3±0.8[c]
SOD	10.2±0.7[a]	11±0.5[a]	15.16±0.8[b]	20.8±1.2[c]	15.6±0.9[b]

*Enzyme activity of Ascorbate peroxidase (APx), GST and Glutathione reductase (GR) is expressed in μmoles min^{-1} g^{-1} FW, CAT and GPx is expressed in nmoles min^{-1} g^{-1} FW and SOD is expressed in U min^{-1} g^{-1} FW. All results are presented as Mean±SD (n = 3). Different alphabet in the same row represents significant difference in the mean values of estimates at 5% level

activity of APx, GST and SOD with no significant change in activity of GPx, GR and CAT. This has suggested the transcriptional regulation of all the antioxidant enzymes in tobacco shoot. In root, APx, GR, GST and GPx enzyme activity pattern was found to be similar to that of respective transcript expression data with both the treatments. However, CAT and SOD activity showed the reverse trend to that of expression pattern in tobacco root (Table 1). This has suggested the transcriptional regulation of APx, GR, GST and GPx and post transcriptional regulation of CAT and SOD enzymes in tobacco roots. Further, results have suggested that higher mRNA production does not always correlate with higher activity. There could be two reasons for this, either the rate of such mRNA degradation is higher or inactive protein enzyme is produced.

Though several independent studies have been conducted documenting the functions of flavonoid and antioxidant systems in plants, studies pertaining to the effect of flavonoids on



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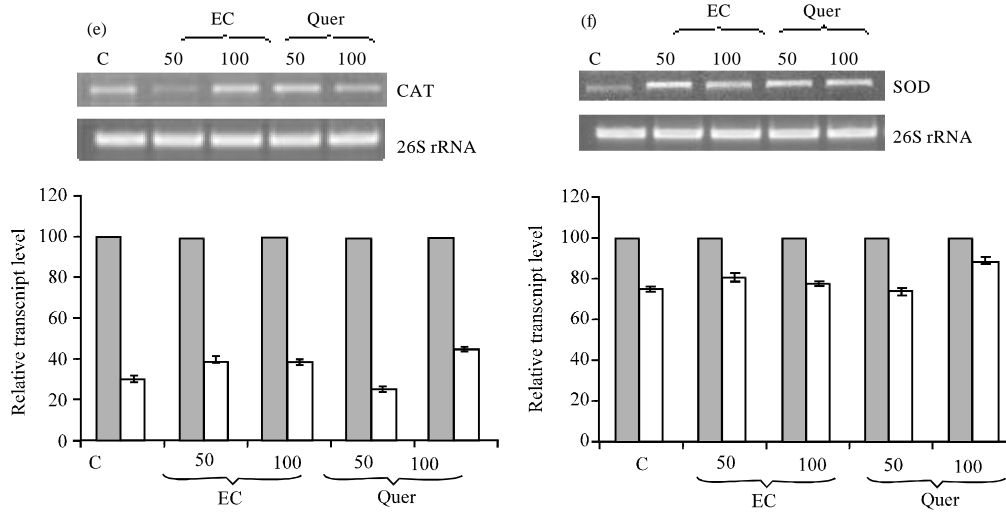

Fig. 2(a-f): Changes in the transcript level of (a) Glutathione peroxidase (GPx), (b) Ascorbate peroxidase (APx), (c) Glutathione reductase (GR), (d) Glutathione S-transferase (e) Catalase (CAT) and (f) Superoxide dismutase (SOD) in tobacco roots in response to epicatechin (EC: 50 and 100 μM) and quercetin (Quer: 50 and 100 μM) different treatments. Bar diagram shows relative transcript levels of the respective amplified bands. Expression analysis was repeated at least three times and representative one time gel pictures are presented. Data are means of three measurements±SD. Black and grey bars showed 26S rRNA and antioxidant enzyme transcript levels, respectively. C: Control, EC: Epicatechin, Quer: Quercetin

antioxidant systems are still lacking. Perhaps one of the greatest challenges is to prove the molecular mechanism(s) through which these flavonoids exert beneficial activity in plants itself. Flavonoids are synthesized mainly in the cytosol through multi-enzymatic complexes that are linked to endoplasmic reticulum membrane (Hrazdina and Wagner, 1985). From the site of synthesis, flavonoids are transported to their subcellular destinations. Owing to highly redox activities of these flavonoids, their subcellular trafficking is tightly regulated to avoid undesired chemical or enzymatic reactions. It is presumed that inside the plants either these flavonoids undergo conjugation with glutathione or they undergo modification by glycosylation and prenylation. Specific flavonoid-conjugate transporters help them to cross the membranes and reach to vacuoles. From vacuole, these flavonoids get remobilised and activate ROS-scavenging genes by binding to the targets or receptors directly or indirectly (Dixon and Pasinetti, 2010).

Furthermore, few biochemical studies have been conducted that showed the interaction of flavonoid and antioxidant pathways. An increased antioxidant activity in phenolics and flavonoids rich fractions of *Convolvulus arvensis* (Elzaawely and Tawata, 2012) and *Vernonia blumeoides* leaves (Aliyu *et al.*, 2011) have been reported. A positive relation between increased flavonoids and antioxidant enzyme activity has been reported in seeds of *Aframomum sceptrum* (Erukainure *et al.*, 2011). The GPx enzyme activity has been reported to be activated by the action of flavonoids, quercetin and catechin (Nagata *et al.*, 1999). Recently, it has been shown through *in vivo* studies that increase in flavan-3-ols (catechin and epicatechin) through downregulation of flavonol synthase enhanced the activity and expression of many antioxidant enzymes in tobacco

(Mahajan *et al*., 2012).The quercetin and its derivatives have been found to prevent oxidative cell damage by either reducing the activity of glutathione peroxidase or increasing glutathione level (Cruz *et al*., 1998). Flavonoids have also been reported to protect cells from glutathione depletion with the cooperation of ascorbic acids (Skaper *et al*., 1997). However, the results of the present study documented that flavonoids might be regulating antioxidant system by acting at transcriptional as well as post-transcriptional levels.

CONCLUSION

The present study has suggested the possible regulation of antioxidant enzymes at transcriptional and post-transcriptional levels by two flavonoids i.e. quercetin and epicatechin. Effect of these flavonoids was found to be concentration dependent and tissue specific. Hence, appropriate levels of these flavonoids are essential for such regulations. Results have documented the effect of flavonoids on the antioxidant system of plants. This study would help in further understanding the cross talk between flavonoid and antioxidant pathways.

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