Polyphenol oxidases (PPOs) are nuclear-encoded copper-containing metalloproteins involved either in hydroxylation of monophenols to o-diphenols (EC 1.14.18.1; monophenol monooxygenase, tyrosinase, and cresolase) or in the dehydrogenation of o-diphenols to o-quinones (EC1.10.3.1, diphenol oxygen oxidoreductase and catecholase). Labile quinones formed from PPO-catalyzed reactions form several co-products. Labile quinones react with each other to yield “melanoid” pigments and react with amino acids/proteins to form brown-colored complexes called tannins/melanins.

PPOs are induced in response to wounds and pathogens and are supposed to play a role in plant defense against insects and plant pathogens. Although role of PPO in plant metabolism is not very clear, quinones produced by PPOs may act as antibiotics and cytotoxins to pathogens. Active quinones covalently modify plant proteins, thus affecting their nutritive value, and increasing the plant’s resistance towards herbivorous insects. Recently, role of PPO in tyrosine metabolism, betalain biosynthesis, lignan biosynthesis, and aurone biosynthesis was described. Although PPOs have been purified and characterized from a large number of plant and fungal species, mushroom tyrosinase is widely used at commercial scale owing to relative ease of production. More importantly, PPO has various industrial applications that extend from food to chemical industry, including the treatment of industrial effluents.

PPOs isolated from various sources have different affinities for different substrates. Substrate affinity depends on the abundance and type of substrate present in a given source and the monophenolase or diphenolase activity of PPO. Substrate affinity of tea PPO toward catechins and gallocatechins plays a very important role in the synthesis of commercially important black tea pigments theaflavins (TFs). TFs are reported for many medicinal properties. Thus, there is a need to develop the heterologous expression system for the production of active PPO to have a continuous source of the enzyme and be independent of the source i.e. tea leaves. Further, the role of PPO in plants is still an enigma. Keeping in view the commercial importance of PPO and that its role in plants is not yet known, the present work was undertaken with the following objectives:

1. Cloning and analysis of PPO from different cultivars of *Camellia sinensis*.
2. Functional validation of cloned genes in heterologous system.
3. Evaluating the possible role of PPO in plants.
Summary

Seven cultivars of tea growing in the experimental tea farm of CSIR-IHBT (32°6’ North latitude; 76°33’ longitude; 1300 m above mean sea level), were used in the present study. Out of seven, 6 cultivars BS-08, BGP-138, CS-08, KD-06, T-253, and T-383 were of China type clones and UPASI-9 was of Assamica type clone.

- **CsPPO** gene from six cultivars, BS-08, BGP-138, Cs-08, KD-06, T-383, and UPASI-9 had open reading frame (ORF) of 1800 bp and each encoded protein of 599 amino acids. **PPO** gene from T-253 had an ORF of 1788 bp encoding a protein of 595 amino acids. The cloned genes had 98-99% identity at the nucleotide level and 91-99% identity at the amino acid level with each other. They were predicted to produce peptides of 66.5-67.3 kDa having pI of 5.98-6.75.

- Derived amino acid sequences had cleavage site for the chloroplast transit peptide between 44th and 45th residues and thylakoid lumen transit peptide cleavage site between 96th and 97th residues. The peptides had targeting sequences for chloroplast lumen and the predicted mature proteins were of 56.2-57.1 kDa with pI of 5.51-6.07. The two conserved copper-binding sites were present; CuA was located at amino acids 204-221, and CuB was located at amino acids 363-374. A third putative Cu binding site was also present at amino acids 531-567.

- **CsPPOs** without chloroplast transit peptide (**CsPPO WCTP**) from different cultivars were cloned into the pQE-30UA vector and expressed in *E. coli* M15[pREP4] cells. However, expression of recombinant protein was not achieved. Cloning of **CsPPO WCTP** into the pET-47b(+) vector and further expression in *E. coli* BL21(DE3) cells also did not yield any recombinant protein. Also, cloning and expression of **CsPPO** gene in the yeast *Kluyveromyces lactis* failed to produce any detectable recombinant protein.

- The lack of expression could be attributed to the presence of high number of rare codons. Thus, **CsPPO** from different cultivars were cloned in pET-47b(+) vector and expressed in *E. coli* Rosetta™ 2 cells containing additional tRNAs of seven rare codons. Although CsPPO protein was expressed in Rosetta™ 2 cells, the expression level was very low.
Therefore, a synthetic codon-optimized \textit{CsPPO} was cloned into pET-47b(+) vector, and its expression in \textit{E. coli} BL21(DE3) cells also yielded recombinant protein in the form of inclusion bodies. Further, lowering of temperature or IPTG concentration could not produce the soluble \textit{CsPPO}.

\textit{CsPPO} inclusion bodies were solubilized in a denaturing buffer containing 8 M urea and attempt to refold the protein were made by various methods such as dialysis, on-column refolding and rapid dilution. Dialysis against 120 mM NaCl or stepwise dialysis in the presence of (NH$_4$)$_2$SO$_4$ did not yield active \textit{CsPPO}. Further, the 6xHis-tagged protein refolding was attempted by immobilized metal ion affinity chromatography. However, protein from Ni-nitritotriacetic acid resin affinity chromatography could not be eluted in the elution buffer.

To resolve the refolding of protein, refolding of \textit{CsPPO} was attempted with rapid dilution in different refolding buffers with additives (glycerol, DTT, NaCl, polyethylene glycol-6000, guanidine hydrochloride, glutathione, and L-arginine) at 15 or 4°C. PPO activity was found to be higher when the refolding was carried out at 15°C as compared to at 4°C. Maximum activity of \textit{CsPPO} was obtained when the inclusion bodies were refolded by dilution in buffer containing 500 mM L-arginine. Although additives such as NaCl, guanidine hydrochloride, glutathione, and glycerol also increased the PPO activity, their effect was much less than L-arginine.

Copper was incorporated in the refolded \textit{CsPPO} and was analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES). The result showed that the estimated copper content was 0.880±0.095 g/g of protein, suggesting that the copper was incorporated in \textit{CsPPO} during refolding, thus showing PPO activity.

Optimum pH for the PPO activity was found to be 5.0. The PPO had $K_m$ of 3.10, 0.479, and 0.314 mM and $V_{max}$ of 163.9, 82.64, and 142.8 U/mg of protein, for catechol, catechin, and epicatechin, respectively.

To understand the potential role of PPO in plants, two year old vegetatively propagated plants of UPASI-9 grown in plastic sleeves were subjected to drought
stress. Samples were collected for estimation of catechins content, PPO activity, and theaflavins content. Results showed that catechins content decreased in drought stressed plants, while PPO activity and theaflavins content were increased as compared to the control plants. Since there was modulation of catechins and theaflavins under drought stress, it was important to understand their effect on biochemical parameters.

- Effect of epigallocatechin gallate (EGCG; most abundant catechin in tea) and black tea extract (BTE; contains >80 % theaflavins) on the activity of tea enzymes namely nitrate reductase (CsNR), glutamine synthetase (CsGS), malate dehydrogenase (CsMDH) and purified porcine MDH (SsMDH) was studied. Both EGCG and BTE inhibited the enzyme activity in a dose-dependent manner. However, EGCG completely inhibited the activity of CsNR, CsGS, CsMDH and SsMDH at 10, 200, 100 and 200 μM, respectively. Whereas BTE completely inhibited the activity of CsNR, CsGS, CsMDH and SsMDH at 5 ngml⁻¹, 50 ngml⁻¹, 20 μgml⁻¹ and 2 μgml⁻¹, respectively.

- Those physical or physiological conditions that would lead to electrolyte leakage, may result in the release of catechins from the vacuole. Catechins per se and their condensed products could interact with the biochemical machinery of the cells. Thus, transgenic Arabidopsis thaliana plants overexpressing CspPO were analyzed for their response to PEG-induced stress.

- Analysis of transgenic Arabidopsis exhibited more rapid progression of PEG-induced programmed cell death (PCD) in transgenic as compared to the wild-type (WT) plants in the presence of externally supplemented EGCG and epicatechin (ECs). This could be due to rapid accumulation of reactive oxygen species as compared to the WT plants.

- Since endoplasmic reticulum (ER) response pathway mediates PEG-induced PCD in Arabidopsis, the expression of stress related genes of ER was studied. Transgenic Arabidopsis plants showed relatively high expression of these genes during PEG-induced stress as compared to the WT plants in the presence of EGCG and EC.
To conclude, CsPPO was cloned from 7 different cultivars of tea. Expression of the protein was achieved only when synthetic gene optimized to eliminate codon bias for expression in *E. coli*. Still, the protein appeared as inclusion bodies, and the active form was achieved after appropriate refolding in buffer containing L-arginine.

Substrates and the products of PPO catalyzed reactions were found to inhibit all the enzymes investigated in the present study (Nitrate reductase, Glutamine synthatase, and Malate dehydrogenase from crude tea extracts as well as purified porcine heart Malate dehydrogenase) suggesting that both catechins and their condensed products are the general inhibitors of enzymes. Furthermore, the work on transgenic *Arabidopsis* showed that PPO promotes PCD during PEG-induced stress in the presence of externally supplied ECs.