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About the Author

Aravind Jukanti obtained both his bachelor's (1993–1997) and master's degrees (1997–1999) in agriculture from Acharya N.G. Ranga Agricultural University, Hyderabad, India. During his master's, his specialization was in "Genetics and Plant Breeding," working on protein and other quality aspects in baby corn maize. Later, he shifted to Montana State University (MSU), Bozeman, USA, on a full scholarship to pursue his PhD in wheat genetics. The title of his dissertation at MSU was "Molecular and Biochemical Characterization of Wheat (*Triticum aestivum* L.) Polyphenol Oxidases." Aravind Jukanti reported and characterized the wheat PPO multigene family. He was also successful in identifying the most important wheat kernel PPO probably involved in the undesirable darkening reaction. After graduating in 2005, he worked as a postdoctoral fellow with Dr. A.M. Fischer at MSU up to 2007, mostly working on grain protein content and nitrogen remobilization in barley. He moved to the lab of Dr. J. Jaworski during the winter of 2007 at Donald Danforth Plant Science Center (DDPSC), St. Louis, Missouri, USA. At DDPSC, he worked on proteomic aspects of triacylglycerol biosynthesis. He moved back to India in 2009; worked in the chickpea team as a breeder at the "International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India"; and later joined the "Indian Council of Agricultural Research (ICAR)" as a "Senior Scientist." He is presently working as a senior rice breeder at ICAR-IIRR, Hyderabad, India.

Introduction 1

Polyphenol oxidases (PPOs) are nuclear-encoded, copper-containing enzymes with diverse phylogenetic distribution among plants, animals, fungi, and bacteria (Yoruk and Marshall 2003; Mayer 2006). In addition to their ubiquitous nature, PPOs are reported in several plant tissues (Vamos-Vigyazo 1981; Sherman et al. 1991, 1995; Yoruk and Marshall 2003). Interestingly, multigene families have been reported for several plant PPOs, but not much is known about their specific biological functions (Anderson et al. 2006). PPOs are often referred to as tyrosinase, phenolase, catecholase, cresolase, polyphenolase, or catechol oxidase (Whitaker 1994, 1996; Yoruk and Marshall 2003). PPOs are also sometimes differentiated into monophenol monooxygenase (EC 1.14.18.1) and diphenol:oxygen oxidoreductase (EC 1.10.3.2) for enzyme nomenclature purposes (Mayer 2006), but in this book, the common term polyphenol oxidase (PPO) will be used. Plant PPOs are implicated in different biological roles, but they are best known for their involvement in the undesirable browning of plant products. The undesirable browning reaction has attracted attention of researchers especially plant breeders, plant physiologists, and food scientists. The oxidation of phenolic compounds by PPOs to o-quinones followed by their nonenzymatic polymerization or condensation with nucleophiles leads to undesirable discoloration of plant products (Mathew and Parpia 1971; Vamos-Vigyazo 1981; Whitaker 1995; Yoruk and Marshall 2003). Interestingly, browning reaction may be useful, viz., in fermentation process of tea (Subramanian et al. 1999) or protein preservation in forage crops (Lee et al. 2004; Sullivan and Hatfield 2006). But mostly the oxidative discoloration observed in several plant-based products is considered negative since they cause deterioration of quality by altering the product's organoleptic and nutritional properties (Vamos-Vigyazo 1981; Mathies and Whitaker 1984; Zawistowski et al. 1991; Martinez and Whitaker 1995). Further, the discoloration of food products negatively impacts the consumer acceptance, storage/shelf life, and their value.

The active site of the enzyme consists of two copper atoms each with three conserved histidine residues (Lerch 1983; Huber et al. 1985). Although a sequential mechanism of binding of substrates is indicated, the order of removal products is

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not fully understood (Wilcox et al. 1985; Janovitz-Klapp et al. 1990; Whitaker 1994). PPOs can catalyze two different reactions: (i) *o*-hydroxylation of monophenols (monophenolase activity) and (ii) oxidation of diphenols to *o*-quinones (diphenolase activity; Mason 1957; Mayer and Harel 1979; Janovitz-Klapp et al. 1990). Some plant PPOs exhibit both types of activities, while others have either of the activities (Yoruk and Marshall 2003). But usually the diphenolase activity is the predominant form in higher plants. Some discrepancies in reaction properties of PPOs have been reported but were later shown to be due to differences in extraction/purification procedures or assay methods utilized (Sanchez-Ferrer et al. 1989b; Wesche-Ebeling and Montgomery 1990; Espin et al. 1997). Accurate determination of PPO activity is very crucial to study its properties and function, since peroxidase (EC 1.11.1.7) also can catalyze the oxidation of *o*-diphenols to *o*-quinones in presence of hydrogen peroxidase (Vamos-Vigyazo 1981; Miller et al. 1990; Nicolas et al. 1994; Richard-Forget and Guillard 1997). Some methods are suggested to detect/reduce the peroxidase interference (Mayer and Harel 1979; Nicolas et al. 1994; Richard-Forget and Guillard 1997). Different methods that quantify oxygen consumption, color formation, and product formation are available to determine the PPO activity (Yoruk and Marshall 2003).

The properties of PPOs have been extensively studied in several plant species, and they vary accordingly. Though the plant PPOs are nuclear encoded, they are plastidial enzymes. The synthesis and transport of plant PPOs to chloroplasts, their actual site, are complex processes, but it has the features of import of nuclear-encoded proteins to their respective organelles (Mayer 2006). Interestingly, the location of fungal PPOs is not yet clear, though it appears to be cytoplasmic. Rast et al. (2003) reported that at least some fungal PPOs are cell wall bound, i.e., in the extracellular matrix. Multiple forms of PPOs have been found in several plant species. These multiple forms exhibit distinct differences in their physicochemical and enzymatic properties. Despite the available evidence, there are some conflicting reports regarding the number of molecular forms of PPOs in some species/tissue. Production of artifacts, interconversion among the PPO forms, hormonal induction, and attachment of phenolic products or carbohydrates could result in multiple isoforms of PPOs (Yoruk and Marshall 2003).

The primary substrates of PPOs are different phenolic compounds; the concentration and types of phenols (mono-/di-/triphenols) vary significantly in plants. Substrate specificity of PPOs also varied with species and variety. Some phenolic substrates like catechol are major substrates in different crops like field bean, apple, and peach (Paul and Gowda 2000; Zhou et al. 1993; Flurkey and Jen 1980). Another important physiochemical property that varies significantly with different factors including plant source is the pH optimum (4.0–8.0). It is interesting to note that some PPOs (field bean, 4.0) show narrow pH optimum, while others (lettuce, 5.0–8.0) have a wider pH range (Yoruk and Marshall 2003). Multiple pH optima have also been observed in some plant species. Temperature is another key factor that considerably affects the PPO activity. The optimum temperature varies widely but is mostly in the range of 20–45 °C with a few exceptions like strawberry and

cucumber (50 °C; Serradell et al. 2000; Miller et al. 1990). Thermal stability of PPOs depends upon several factors including temperature and exposure time.

Several of the plant and fungal PPOs characterized were observed to be latent (van Gelder et al. 1997; Mayer 2006), and these are not involved in catalyzing phenols. The extent of latency of plant PPOs varies widely with species and tissues. The latent PPOs can be activated by different methods, like acid and base treatment (Kenten 1957), frost and aging (Lieberei and Biehl 1978, Meyer and Biehl 1980), alcohols (Guillard and Richard-Forget 1997; Espin and Wichers 1999; Onsa et al. 2000), mild heat treatment (Sheptovitsky and Brudvig 1996), and exposure to strong detergents (van Gelder et al. 1997; Chazarra et al. 2001; Okot-Kotber et al. 2002; Jukanti et al. 2003). Sodium dodecyl sulfate (SDS) has been extensively used for activation of latent PPOs in several plant species (Sanchez-Ferrer et al. 1989a, b, 1990; Moore and Flurkey 1990; Jimenez and Garcia-Carmona 1996; Laveda et al. 2000; Jukanti et al. 2003). Additionally, PPOs (both plant and fungal) have also been activated by partial proteolytic degradation (Gandía-Herrero et al. 2005; Jukanti et al. 2006; Mayer 2006). Several studies were conducted to understand the mechanism of proteolytic activation (Robinson and Dry 1992; Rathjen and Robinson 1992; Dry and Robinson 1994). The molecular weight of different forms of plant PPOs is in the range of 32–200 kDa but majority of them in the range of 35–70 kDa (Flurkey 1986; Sherman et al. 1991; Van Gelder et al. 1997; Yoruk and Marshall 2003).

Enzymatic browning caused by PPOs has significant economic impact on plant products (cereal, fruit, and vegetables) and seafood like shrimp/lobsters, thereby necessitating its control. Over the years, several compounds and approaches have been identified that control or prevent the enzymatic browning. Though several inhibitors or approaches are available, their effectiveness varies; therefore, specific control is required for a particular/individual PPO system (Ferrar and Walker 1996). Physical treatments like heating, freezing, and refrigeration could be used as alternative methods to control adverse browning (Ashie et al. 1996; Kim et al. 2000). Food safety is a major concern while using inhibitors to control browning reaction as some of them could be harmful. Therefore, identification of natural inhibitors specifically in the case of fruits and vegetables is very important. Some natural inhibitors like honey (Chen et al. 2000) and few others have been shown to be promising, but they are yet to be used on a commercial scale. But controlled level of PPO expression through DNA-/RNA-based strategies is the safest and most promising approach to reduce/control the browning reaction without any adverse health concerns. Advanced molecular tools have been utilized in manipulating the expression levels of PPOs; these studies will be discussed in detail in other chapters.

A role in plant defense is often been suggested for plant PPOs due to their induction upon wounding, pathogen attack, or insect infestation in addition to various abiotic/biotic stresses or various signaling compounds (Constabel et al. 1995; Thipyapong and Steffens 1997; Maki and Morohashi 2006). Despite PPOs' implication in plant defense mechanisms, most part of the early research focused on correlative studies. But recent progress has enabled in deciphering and understanding at least some molecular mechanisms of PPO action in important functions including plant defense (e.g., Thipyapong et al. 2004a). With recent advances in molecular

techniques, it is now possible to study/examine the role of specific PPO genes in response to injury, pest/pathogen attack, or abiotic stresses. Further, in some recent studies, the PPO expression levels were manipulated to study the PPO action or mechanisms to pathogen attack, water stress, and salinity (Thipyapong et al. 2004a [plant defense], b [water stress]; Liang et al. 2006). Induction of PPO has also been reported in fungi upon bacterial infection (Soler-Rivas et al. 2000). There is no doubt that these studies have given some insights of PPO action, but there is still no direct explanation for the underlying mechanism(s).

In plants, PPOs are mostly known for their role in unacceptable browning of products and to some extent for involvement in plant defense. However, precisely what roles do these enzymes play in plant metabolism is still very unclear. Majority of the characterized plant PPOs have diphenolase/catechol oxidase activity, but some have monophenolase/tyrosinase activity (Sullivan 2015). Though it was suggested that PPOs could be involved in production of caffeic acid from *p*-coumaric acid due to the tyrosinase activity (Vaughan and Butt 1969), it was later proved to be otherwise (Schoch et al. 2001; Franke et al. 2002). However, due to the occurrence of multiple forms of PPOs in several species, it is possible that PPOs are capable of performing important roles in plant metabolism. Some recent studies have demonstrated the possible role of PPOs in specific cases including (i) betalain biosynthesis (Gandia-Herrero and Garcia-Carmona 2013), (ii) tyrosine metabolism (Araji et al. 2014), (iii) lignin biosynthesis (Cho et al. 2003), and (iv) aurone biosynthesis (Sato et al. 2001). Additionally, the availability of different advanced analyses (genomic, transcriptomic, metabolomic, and proteomic) can help in studying and understanding the role(s) and function of PPOs in plants and other organisms.

In addition to plants, PPOs are also reported in seafood products like shrimp (Simpson et al. 1988; Rolle et al. 1991; Chen et al. 1997) and lobster (Chen et al. 1991a; Ali et al. 1994). Enzymatic browning also called as melanosis is a major concern in these economically important products as they are highly vulnerable to browning reaction. PPO inhibition studies have been a major area of research in the seafood products (Chen et al. 1991b, 1993; Kim et al. 2000). The seafood like shrimp is treated with chemical preservatives/melanosis inhibitors (e.g., sulfites) in the processing facilities to control the undesirable enzymatic reaction and extend their shelf life (Montero et al. 2001). Kim et al. (2000) described PPOs in seafood and their impact on quality. Animal PPOs are involved in the biosynthesis of melanin, the pigment of hair and skin (Hill 1992). Additionally, they are responsible for cuticular hardening, wound healing, and defense reactions in crustaceans and insects (Ferrer et al. 1989; Gillespie et al. 1997; Sugumaran 1998).

The first report of PPOs was by Bertrand (1896), and since then there have been several hundred papers addressing different aspects of PPOs. Detailed work on PPOs was made possible due to the pioneering work of Keilin and Mann (1938) and Kubowitz (1938) with regard to enzyme isolation. Later it was Mason (1956, 1966) who described the structure, possible functions, and importance of number of copper atoms at the active site. He also stated the necessity to understand the nature of PPO isozymes and monophenolase/diphenolase activity. Now it is an established fact that both monophenolase and diphenolase activities are the features of PPOs.

Several comprehensive reviews on plant PPOs including Mayer and Harel (1979), Steffens et al. (1994), Yoruk and Marshall (2003), and Mayer (2006) have been published. The authors have reviewed and discussed the various attributes including functions, structure, multiplicity, induction, and molecular properties of PPO. The latest review by Sullivan (2015) discussed the specific role of PPOs in biosynthesis of metabolites through both monophenolase and diphenolase activities. Although significant new observations regarding the different aspects of PPOs have been elucidated, certain problems like the function (including the mechanism), location, expression, in vivo activation, and molecular control have not yet been unambiguously resolved. Hence, the major focus of future work should be to understand the mechanisms/function and comparatively less to studies on presence of PPOs in a species. The use of advanced biological tools coupled with newer approaches (transcriptomic, proteomic, and metabolomic) will definitely aid in effectively addressing the pertinent problems of PPOs in the near future. In this book, I have made a sincere effort in presenting the different attributes of PPOs in a most comprehensive way by including the latest findings about plant PPOs in particular.

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Distribution, Localization, and Structure of Plant Polyphenol Oxidases (PPOs)

2

The plant PPOs are an intriguing enzyme system present across the plant kingdom potentially involved in a wide variety of roles and functions. Most significantly, the plant PPOs are known for their role in the undesirable browning of plant products. The *o*-quinones, reaction product of PPOs, are responsible for the formation of dark brown coloration of plant products. The multiple nature of plant PPOs could be due to their involvement in diverse roles including plant defense and pigment formation. Further, the wide distribution coupled with presence of multiple genes obviously necessitates wide variability in the physical and biochemical properties to perform multiple functions in different plant species under variable conditions.

2.1 Distribution and Localization of Plant PPOs

The list of plant species in which PPOs have been identified and characterized (at least partially) is certainly growing (Table 2.1). But information obtained in majority of the studies do not widen/expand our understanding of this enzyme. Majority of studies give details about molecular weight and multiplicity of PPOs; very few reports about special features are available. Apart from fruits, vegetables, and cereals, PPOs have also been reported from some unusual and exotic plant species. Four plastid-localized PPO isoforms were purified and partially characterized from aerial roots of *Aranda*, an orchid (Ho 1999). PPOs have also been reported from lesser-known plant species like marula (*Sclerocarya birrea*; Mduli 2005), persimmon (*Diospyros kaki*; Navarro et al. 2014; Núñez-Delicado et al. 2003), soursop (*Annona muricata*; Bora et al. 2004), oregano (*Origanum vulgare* spp. Hirtum; Dogan et al. 2005a), loquat (*Eriobotrya japonica*; Sellés-Marchart et al. 2006), artichoke (*Cynara scolymus*; Dogan et al. 2005b; Quarta et al. 2013), dodder (*Cuscuta* sp.; Bar Nun and Mayer 1999), and *Uapaca kirkiana* (Muchuweti et al. 2006). Interestingly, Tran et al. (2012) have identified only two PPO genes in rice (*Oryza sativa*) and none in *Arabidopsis thaliana* (thale cress) or *Arabidopsis lyrata* (lyrate rockcress). The absence of PPO genes in *Arabidopsis* sp. was also reported earlier

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Table 2.1 Putative number of PPO genes in different plant species[a]

S. no	Common name	Scientific name	PPO genes
1	Rice	*Oryza sativa*	2
2	Maize	*Zea mays*	6
3	Cereal grass	*Sorghum bicolor*	8
4	Foxtail millet	*Setaria italica*	4
5	Barley	*Hordeum vulgare*	2
6	Wheat	*Triticum aestivum*	6
7	Tomato	*Solanum lycopersicum*	7
8	Potato	*Solanum tuberosum*	5
9	Eggplant	*Solanum melongena*	6
10	Cucumber	*Cucumis sativus*	1
11	Soybean	*Glycine max*	11
12	Cassava	*Manihot esculenta*	1
13	Apple	*Malus x sylvestris* (var. *domestica*)	2–4
14	Grapevine	*Vitis vinifera*	4
15	Peach	*Prunus persica*	4
16	Papaya	*Carica papaya*	4
17	Pineapple	*Ananas comosus*	3
18	Castor bean	*Ricinus communis*	1
19	Barrel medic	*Medicago truncatula*	4
20	Black poplar	*Populus trichocarpa*	11

[a]Adapted from Taranto et al. (2017) and Tran et al. (2012)

by Van der Hoeven et al. (2002). Further, candidate PPO genes have also been identified among several plant genomes from four distantly linked lineages including bryophytes, lycophytes, monocotyledonous anthophytes (monocots), and eudicotyledonous anthophytes (eudicots; Table 2.1; Tran et al. 2012).

The subcellular location of PPOs is very crucial for ascribing a physiological role to the enzyme; therefore, the location of the PPO in plants has always attracted the attention of the researchers in the field. Majority of PPOs are plastid localized in higher plants (Mayer 1987; Mayer and Harel 1979; Tolbert 1973). PPOs are usually located in the thylakoid membrane of chloroplasts and in vesicles of other non-green plastids (Nicolas et al. 1994; Vaughn et al. 1988). It appears that PPO is synthesized on the cytoplasmic ribosomes and is probably integrated in inactive form into the plastid (Yoruk and Marshall 2003). Later, a chaperone-stabilized protein might be translocated and integrated into the thylakoid membrane (Yalovsky et al. 1992; Marques et al. 1995). The nuclear-encoded precursor PPOs containing a transit peptide sequence at the amino-terminus end are targeted to the chloroplast. This precursor form is transported into the chloroplast only after the removal of the signal sequence by stromal peptidases (Koussevitzky et al. 1998). During this process of incorporation, the high molecular weight, inactive precursor form (~65 to 68 kDa) is transformed into mature, low molecular weight protein (~60 kDa; Sommer et al. 1994). This observation has been reported in several species including apple (Boss et al. 1994), apricot (Chevalier et al. 1999), grape (Dry and Robinson 1994), and

potato (Hunt et al. 1993). Interestingly, tomato PPO is translocated into thylakoid in two steps: first, the 67-kDa protein is processed to 62 kDa by stromal peptidases in stroma, and this intermediate form is further processed to a 59-kDa mature protein in lumen (Sommer et al. 1994).

Early studies by Arnon (1948, 1949) in spinach beet indicated exclusive localization of PPOs to chloroplast. PPO is assumed to be tightly bound to the thylakoid membrane (Marques et al. 1994; Sheptovitsky and Brudvig 1996; Yoruk and Marshall 2003). But studies have also indicated the existence of PPOs in mitochondria or as soluble/non-membrane bound (Nicolas et al. 1994; Söderhäll et al. 1985), cell walls (Palmer 1963; Barrett et al. 1991), microsomes (Mayer and Harel 1979), or cytosol (Coombs et al. 1974). Existence of both membrane (chloroplast/mitochondria) and non-membrane-bound PPO is shown in apple fruit (Demenyuk et al. 1974; Harel et al. 1964). The non-membrane-bound or soluble fraction of PPO increases during apple fruit ripening (Barrett et al. 1991; Harel et al. 1964) probably due to release of plastidial form to the cytoplasm during ripening and senescence (Lieberei et al. 1981; Barrett et al. 1991). In tomato PPO gene family, only three PPOs possessed the hydrophobic character required for membrane association, and the other four members lacked the necessary domains (Newman et al. 1993).

The oxidation of phenolic compounds by PPOs is undoubtedly their most common role. But the browning is not usually observed in healthy plant tissues perhaps because of the physical compartmentation of PPOs (plastids) and their substrates (phenols) in the vacuoles (Vaughn and Duke 1984; Vaughn et al. 1988). The physical barrier is overcome either by senescence or injury causing the interaction of PPO with its phenolic substrates and resulting in the dark brown discoloration of the tissue (Vaughn and Duke 1984; Felton et al. 1989; Murata et al. 1997; Thipyapong et al. 1997). However, it is still to be identified if there are other factors besides injury and membrane disruption, which are involved in the activation of enzymes that would be capable to oxidize phenolic substrates. Further, whether these factors will affect the enzyme quantity and activity levels available during different developmental processes including senescence needs further investigation.

The identification of six genes coding for PPO in hexaploid wheat, of which at least three are expressed during kernel development (Jukanti et al. 2004), is worth noting. Studies in wheat have revealed that key genes controlling PPO activity are found on wheat chromosome 2 (Udall 1996; Jimenez and Dubcovsky 1999; Anderson and Morris 2001; Demeke et al. 2001; Mares and Campbell 2001), but additional genes present in group 3 (Udall 1996; Demeke et al. 2001), group 5 (Udall 1996), and 6B and 7D chromosomes (Li et al. 1999) could modulate or influence the PPO activity. Raman et al. (2005) identified a major locus controlling PPO activities on the long arm (2AL) of chromosome 2A that explained 82–84 % of the genetic variation, using a QTL-based approach in a doubled haploid population derived from Chara/WW2449. In barley, the PPO gene has been genetically mapped to the long arm of barley chromosome 2H (Takeda et al. 2010). Similarly, in grape four PPO homologues were located on chromosome 10 (Virador et al. 2010).

Table 2.2 Plant PPO sequences used in analysis

S. no	Common name	Scientific name	GI number of protein	Abbreviation used
1	Pineapple	*Ananas comosus*	13559508	AcPPO
2	Sweet potato	*Ipomoea batatas*	29691902	IbPPO
3	Tomato	*Lycopersicon esculentum*	1172583	LesPPO
4	Tobacco	*Nicotiana tabacum*	2916727	NtPPO
5	Japanese rice	*Oryza sativa* subsp. *japonica*	38344179	OsPPO
6	Potato	*Solanum tuberosum*	1146424	StPPO
7	Spinach	*Spinacia oleracea*	1172585	SoPPO
8	Bread wheat	*Triticum aestivum*	46946546	TaPPO
9	Red clover	*Trifolium pratense*	2902363	TpPPO
10	Broad bean	*Vicia faba*	1172586	VfPPO
11	Wine grape	*Vitis vinifera*	1172587	VvPPO
12	Giant octopus	*Octopus dofleini*	15988083	OdPPO

Adapted from Marusek et al. (2006)

2.1.1 Structure of Plant Polyphenol Oxidase

The plant PPOs are nuclear-encoded copper-containing enzymes. The catalytic site of all PPOs contains a dinuclear copper center with two copper ions; each of the copper ions is coordinated by three histidine residues (Gerdemann et al. 2002). The copper center binds to molecular oxygen in a side-on bridging binding mode (details are explained in the succeeding paragraphs). Plant PPOs contain a bipartite amino (N)-terminal transit peptide fragment that directs the PPO to the thylakoid lumen of the chloroplast (Steffens et al. 1994). Further, within the thylakoid lumen, PPOs are either soluble or associated with the thylakoid membrane. Fungal PPOs do not contain the transit peptide and are assumed to be cytoplasmic, probably bound to organelles or membranes (Jolivet et al. 1998; Rast et al. 2003). PPOs are mostly present as 55–70-kDa latent forms which undergo processing or cleavage by ~15 to 20 kDa at the carboxy (C)-terminal end to yield an active enzyme (van Gelder et al. 1997). But molluskan hemocyanins (HCs) do not undergo cleavage at the C-terminal end and function as a large, multi-subunit structure (Marusek et al. 2006). Depending upon the species, ten or more subunits assemble to form cylindrical structures (Lambert et al. 1994; Mouche et al. 1999; Meissner et al. 2000).

Marusek et al. (2006) showed several important structural features that are conserved across various plant PPOs using sequence alignments and secondary structure predictions. They have compared 11 plant species as listed in Table 2.2. All the species possessed the six histidine residues that coordinate with the two copper ions of the catalytic center. An unusual thioether bridge was observed in IbCo and OdHC crystal structures present between a cysteine and the second histidine of the CuA site (Cuff et al. 1998; Klabunde et al. 1998). Since the cysteine was very well conserved in all the 11 plant species analyzed, it was speculated that all plant PPOs have a thioether bridge as part of their CuA sites (Marusek et al. 2006). Further, the authors suggest that the thioether bridge is important (may not be essential) as a

structural and component of the catalytic site of type 3 copper proteins including plant PPOs. Another conserved feature observed was the Phe261 (gate residue), which partially blocks the active site (Gerdemann et al. 2002). The sequence alignments show that majority of plant PPOs have phenylalanine as a gate residue except AcPPO and TaPPO which have smaller leucine similar to as in OdHC (Decker and Tuczek 2000).

García-Borrón and Solano (2002) observed a pair of tyrosines conserved in PPOs from organisms including humans. The sequence alignment studies of plant PPOs revealed that the first tyrosine could be substituted by a phenylalanine as in LesPPO, NtPPO, and OsPPO (Table 2.2; Marusek et al. 2006). This conserved sequence, Y/FxY, was referred to as "tyrosine motif" and is located in the CuB site. The first tyrosine of the tyrosine motif is involved in a pi–cation interaction with arginine located near the N-terminus. Interestingly, this arginine is also conserved in all the 11 plant species evaluated. The pi–cation interaction could probably be responsible for the overall structural integrity of the N-terminal domain as evidenced by the conservation of residues involved in the interaction. The residues following the tyrosine motif form the beginning of the peptide chain that lead to the globular C-terminal domain. Majority part of this peptide fragment joining N- and C-terminal domains is surface-exposed alpha-helix, and this connecting fragment is referred to as "linker region." Thus, the tyrosine motif is a landmark in all plant PPOs as it indicated the end of N-terminal globular domain. Further, the length of amino acid sequence prior to CuA and the sequence between CuA and CuB sites vary indicating some minor structural differences. Overall, the N-terminal structure with the conserved features among several plant PPOs indicates that they might have a similar architecture.

Marusek et al. (2006) predicted and compared the secondary structures of sequence following the tyrosine motif of all the 11 PPOs with that of OdHC. The six beta-strands were numbered from 1 to 6 in order as they occur in the primary amino acid sequence. All the 11 PPOs have a predicted secondary structure similar to that of OdHC. Leu2830 of OdHC C-terminal domain could be an important residue since it is the only residue in direct contact with the active site (Cuff et al. 1998), and its side chain blocks substrate access to the catalytic site. This residue was proposed to act as "sensor" of active site events (Cuff et al. 1998). The corresponding residue in plant PPOs is leucine, isoleucine, valine, or alanine. As mentioned above, the sequence joining N- and C-terminal domains is called as "linker" region, and the length of the linker region varies from 50 to 80 residues in plant PPOs as against 24 in OdHC. The site of C-terminal processing is located within/middle of the linker region (Robinson and Dry 1992; Flurkey 1989; Dry and Robinson 1994).

The ~15 to 20 residues at the end of the linker region and just prior to the beta-strand 1 of the C-terminus were predicted to be disordered in all plant PPOs (Marusek et al. 2006). The authors speculate that this region is disordered under certain conditions and becomes ordered under different conditions. Additionally, two other short regions of disorder near the beginning of the linker region and in the loop connecting beta-strands 3 and 4 were also identified in most of the plant PPOs. Marusek et al. (2006) also identified a conserved serine residue in the linker region that is probably

phosphorylated in all the plant PPOs. The phosphorylation site location is consistent with that of a residue in the C-terminal fragment of SoPPO (Race et al. 1995). The phosphorylation site of the plant PPOs is located in part of the linker region predicted to be disordered. Despite the prediction of a potential *N*-β-GlcNAc glycosylation of an asparagine and *O*-β-GlcNAc to serine and threonine residues in certain plant species, it is difficult to say with a certainty that these are true glycosylation sites. The authors have demonstrated many important structural features of several plant PPOs including copper-coordinating histidine residues, thioether bridge, the gate residue, and tyrosine motif probably present in all the plant PPOs. Several of the plant PPOs have a linker region, connecting the N- and C-terminal domains. The authors also speculate that disordered linker region containing the probable phosphorylation site could be crucial site of in vivo activation.

2.2 Crystal Structure of Plant PPOs

2.2.1 Structure of Sweet Potato (*Ipomoea batatas*) Catechol Oxidases

Catechol oxidase (PPO), hemocyanin, and tyrosinase are dicopper proteins that contain a coupled dinuclear metal center (Klabunde et al. 1998). This type 3 copper center binds two oxygen molecules in a "side-on" bridging binding mode resulting in oxygen activation. The distinctive spectral features of the oxygenated forms of these dicopper proteins include (i) the electrons present in the two "Cu" atoms that have a strong antiferromagnetic coupling between them, (ii) a low-energy O–O stretching vibration at 750 cm^{-1} in the resonance Raman spectrum, and (iii) absorption spectrum maxima with charge transfer bands [$O^{2-}{}_2 \rightarrow$ Cu(II)] at ~350 nm [E ~ 20,000 M^{-1} $cm^{-}1$] and ~600 nm [E ~ 1000 M^{-1} $cm^{-}1$] (Solomon et al. 1996; Magnus et al. 1994; Eicken et al. 1998). Though these proteins have similar geometric and electronic structures of their respective dicopper centers, their biological functions differ: hemocyanin transports oxygen and acts as a storage protein in arthropods and mollusks; tyrosinase catalyzes hydroxylation of phenols and oxidation of catechol product to quinone (Lerch 1983).

Catechol oxidase is also called as polyphenol oxidase or *o*-diphenol oxidase (EC 1.10.3.1). Though it lacks hydroxylase activity, it catalyzes a two-electron transfer reaction during oxidation of *o*-diphenols to corresponding *o*-quinones (Cary et al. 1992). These *o*-quinones as discussed earlier form brown-colored melanins, thought to be involved in plant defense against pest and pathogen attack (Dervall 1961). In the three-dimensional crystal structure of catechol oxidase (PPO) from sweet potato (*Ipomoea batatas*) in oxidized state (Cu[II]–Cu[II]) and reduced form (Cu[I]–Cu[I]) and as a complex with phenylthiourea (PTU), its inhibitor has been determined (Klabunde et al. 1998). The monomeric form (39,000 M_r) of sweet potato catechol oxidase is ellipsoid with dimensions of 55 × 45 × 45 Å (Figs. 2.1a and 2.1b).

The secondary structure is largely α-helical with a four-helix bundle composed of α-helices (α2, α3, α6, and α7) forming the core of the enzyme. The dinuclear

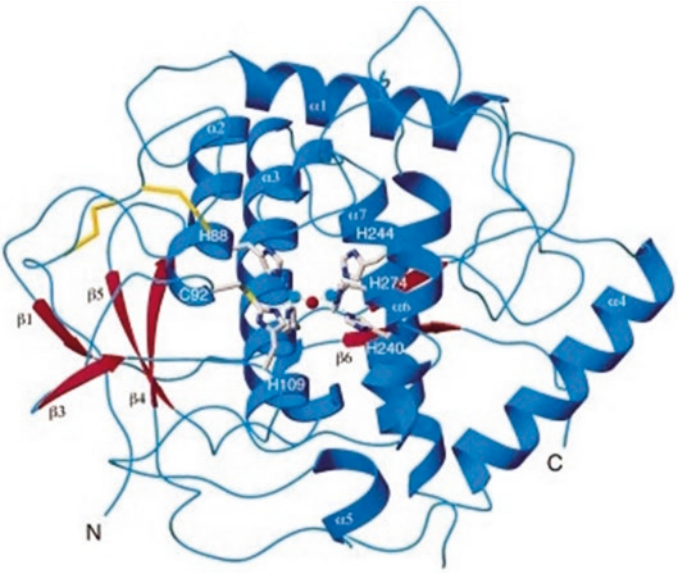

Fig. 2.1a Ribbon drawing of the frontal view of the 39,000-M_r catechol oxidase (Source: Klabunde et al. 1998)

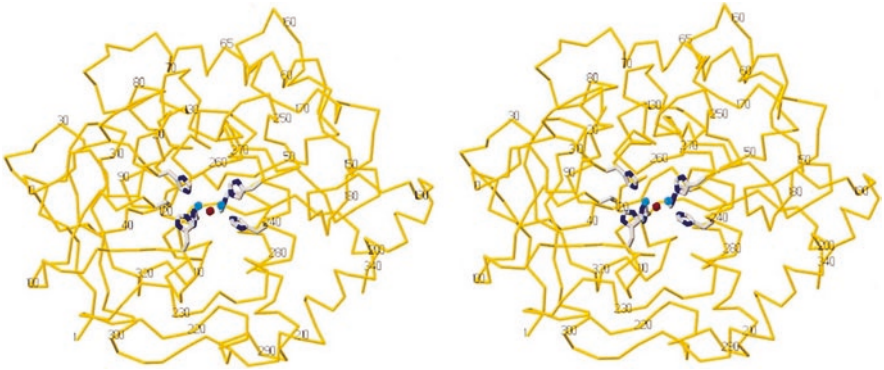

Fig. 2.1b A stereo diagram of the C_α trace of the enzyme. The dicopper site found in the center of the four-helix bundle motif is displayed in stick presentation. Side chains of ligands to the catalytic Cu(II)-OH-Cu(II) unit present in the resting oxidized enzyme form are colored by atom type [carbon is *gray*; nitrogen is *blue*; sulfur is *yellow*] (Source: Klabunde et al. 1998)

copper center is accommodated in the helical bundle, and the center is surrounded by α1 helix, α2 helix, and several short β-strands. The loop-rich amino terminal region of the enzyme (residues 1–50) is anchored to the α2 helix by two disulfide bridges (Cys11–Cys28; Cys27–Cys89). Further, three histidine residues contributed by α2, α3, α6, and α7 helices coordinate each of the two catalytic coppers (Fig. 2.2). The CuA is coordinated by His88 (present in middle of α2 helix), His109

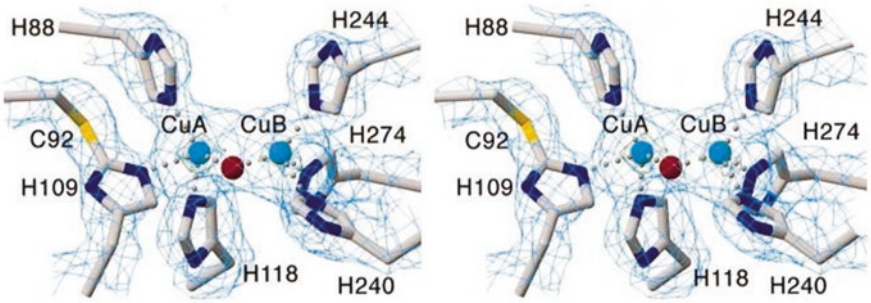

Fig. 2.2 Coordination of the active site coppers by three histidine residues. Both copper sites, CuA on the *left* and CuB on the *right*, show a trigonal pyramidal coordination sphere formed by three histidine ligands and the bridging solvent molecule. A sample of 2 $|F_o| - |F_c|\Phi_{calc}$ electron density, for the oxidized catalytic dinuclear copper site (Source: Klabunde et al. 1998)

(beginning of α3 helix), and His118 (middle of α3 helix). Similarly, CuB, the other catalytic copper, is coordinated by three residues (His240, His244, and His274), located in the middle of helices α6 and α7.

The two cupric ions are 2.9 Å apart in the oxidized catechol oxidase structure, and upon reduction, the metal–metal separation increases to 4.4 Å. The occurrence of covalent thioether bond between the Cε atom of His109, one of the copper ligands, and the sulfur atom of Cys92 is an interesting feature of the dinuclear copper center of the catechol oxidase. Interestingly, thioether linkage has also been reported in tyrosinase from *Neurospora crassa* (Lerch 1982), hemocyanin of molluskans (Gielens et al. 1997; Miller et al. 1998), and octopus (*Octopus dofleini;* Cuff et al. 1998). It is observed that this covalent bond puts additional structural restraints on the CuA center. Further, the restrained geometric structure could impose the observed Cu(I) geometry on the Cu(II) in CuA site, thereby optimizing the electronic structure of the copper required for oxidation of *o*-diphenol and may also allow for a rapid electron transfer in the redox process. The thioether linkage prevents the shifting of His109 and thereby any bidentate binding mode of substrate.

A hydrophobic pocket lined by the side chains of Ile241, Phe261, His244, and Ala264 centrally houses the dinuclear copper site (Fig. 2.3). Aromatic ring of the Phe 261 present in the loop region connecting α6 and α7 helices controls the access to the active site. Binding of an inhibitor like PTU causes conformational change in the phenyl ring of Phe261 and imidazole ring of His244 and forms hydrophobic interactions with the aromatic ring of PTU. Further, the replacement of hydroxo-bridge present in the Cu(II)–Cu(II) enzyme by sulfur of PTU increases the metal–metal separation to 4.2 Å (Fig. 2.3). A weak interaction between amide nitrogen and CuB center is observed. Additionally, high affinity of PTU with enzyme is observed due to the van der Waals interactions between the residues lining the hydrophobic pocket.

Catechol oxidases and hemocyanins share remarkably identical catalytic core, but there is neither significant sequence similarity nor any resemblance in their overall fold. The dicopper centers of catechol oxidases and hemocyanins from

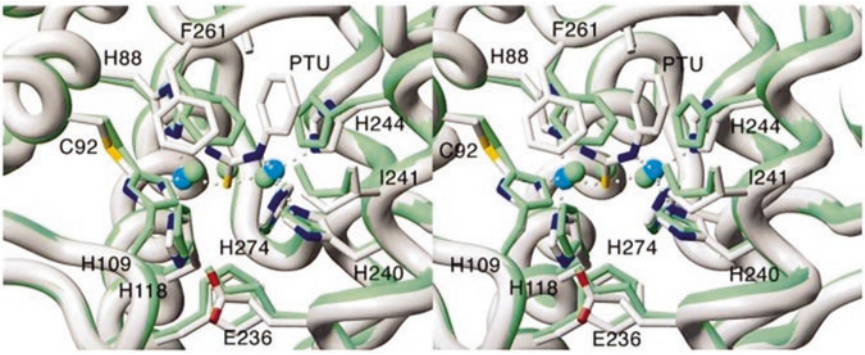

Fig. 2.3 View of the active site region with phenylthiourea bound to the dicopper center. The sulfur of the inhibitor binds to both copper ions. A stick presentation of the active site residues of the resting Cu(II)–Cu(II) state of the enzyme is superimposed in *light green* to reveal the conformational change induced by the binding of PTU (Source: Klabunde et al. 1998)

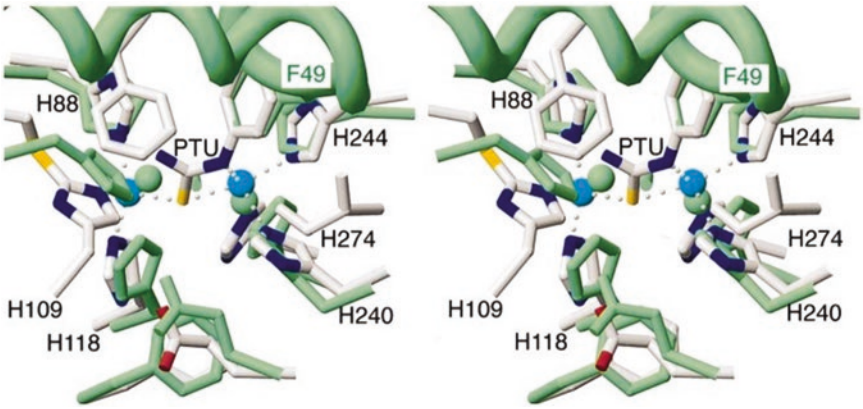

Fig. 2.4 Superposition of the dinuclear copper center of sweet potato catechol oxidase with bound phenylthiourea (PTU) with the oxygenated form of *Limulus polyphemus* hemocyanin. The side chains of catechol oxidase are colored by atom type, and the metal-ligating histidine residues of lpHC are shown in *green* (Source: Klabunde et al. 1998)

Panulirus interruptus (piHC) and *Limulus polyphemus* (lpHC) are similarly buried in the four-helix bundle, and all the six coordinating histidine residues are provided by these four α-helices (Gaykema et al. 1984; Volbeda and Hol 1989; Hazes et al. 1993). An interesting feature of the hemocyanin protein is the shielding of access to the active site by aromatic ring of a phenylalanine (Phe49 for lpHC; Phe75 for piHC; Fig. 2.4) present at the N-terminal region. The aromatic ring of the phenylalanine aligns exactly with aromatic ring of PTU in the catechol oxidase–PTU complex. This shielding of the active site prohibits the binding of substrates and thereby allows the hemocyanins to act as oxygen-carrying proteins.

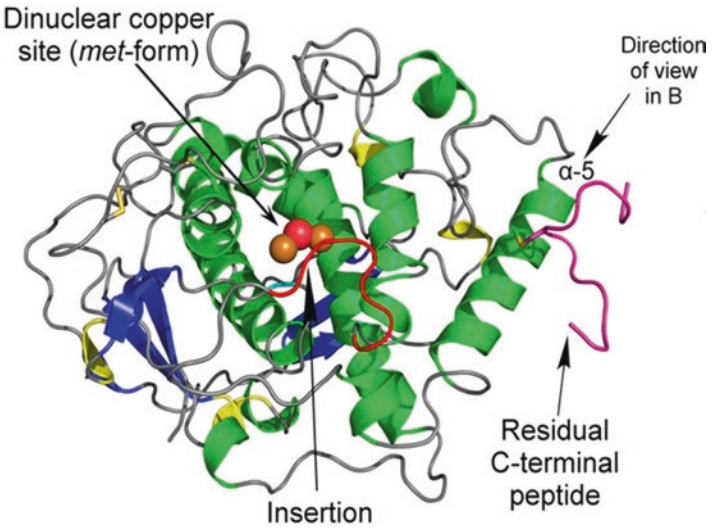

Fig. 2.5a Mature aurone synthase; α-helices, *green*; 310-helices, *yellow*; β-sheets, *blue*; and the characteristic features of AUS1 are colored *red* [loop carrying the insertion $V^{237}ANG^{240}$] and magenta [residual C-terminal peptide] (Source: Molitor et al. 2016)

2.2.2 Structure of Tickseed (*Coreopsis grandiflora*) Aurone Synthase (*Catechol Oxidase*)

Catechol oxidases and tyrosinases are PPOs that belong to type 3 copper protein family. Since catechol oxidases are unable to hydroxylate tyrosinase substrates like tyrosine and tyramine, they were assumed to lack monophenolase activity and possess only diphenolase activity, i.e., oxidation of *o*-diphenols to *o*-quinones. Aurone synthase (AUS1), a PPO homolog, was shown to be involved in the 4-deoxyaurone biosynthesis in petals of tickseed (*Coreopsis grandiflora*; Kaintz et al. 2014; Molitor et al. 2015a). Interestingly, AUS1 exhibited hydroxylase activity toward its natural substrate, isoliquiritigenin, demonstrating that the hydroxylase activity is not linked to the acceptance of usual tyrosinase substrates. Recently, the crystal structure of latent pro-PPO, its mature active and inactive form, has been determined for AUS1 (Molitor et al. 2016). The mature AUS1 (41.6 kDa) crystallized into two space groups ($P2_12_12_1$ and $P12_11$) with four monomers each in an asymmetric unit (Molitor et al. 2015b). Mature AUS1's structure had significant similarity with catechol oxidase (~47 %) from sweet potato (*Ipomoea batatas*; Klabunde et al. 1998) and grapevine (*Vitis vinifera*; Virador et al. 2010) as well as with tyrosinase (~48 %) from walnut (*Juglans regia*; Bijelic et al. 2015). But the AUS1 has certain unique features including a C-terminal peptide (Asp338 to Gly452) that is connected to the active site core by a disulfide bridge (Cys206–Cys445) and formation of cavity near the active site by insertion of four amino acids ($V^{237}ANG^{240}$; Figs. 2.5a, 2.5b and 2.5c).

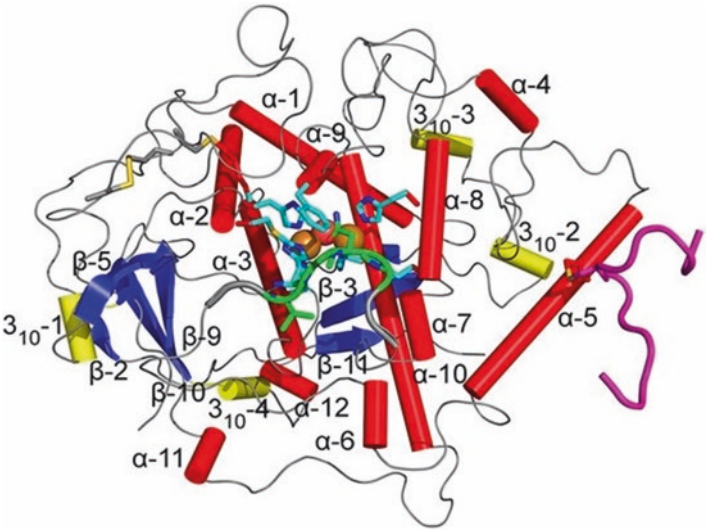

Fig. 2.5b Secondary structure features of mature AUS1 [α-helices, *red* tubes; 3_{10}-helices, *yellow* tubes; β-strands, *blue*] (Source: Molitor et al. 2016)

Both crystal units had homodimeric assemblies of homomers (Krissinel and Henrick 2007), and exclusion chromatography indicated the existence of a dimer in solution. The residual C-terminal fragment with unknown function forms the key building block of the homodimeric interface. The stabilization of the AUS1's quaternary structure is achieved through four hydrogen bonds, several hydrophobic interactions, and 11 bridging water molecules between the residues of C-terminal fragment of a monomer and the α-helix of another monomer that carries the C-terminal peptide.

Latent AUS1 is a monomer, and the structure of the main core of latent AUS1 is identical to that of the mature AUS1 except for the location of a single loop (has characteristic insertion of $V^{237}ANG^{240}$) that gives way for the shielding C-terminal domain (Fig. 2.6).

The C-terminal domain starts with a short linker region of ~10 amino acids located close to the interdomain disulfide bridge (Fig. 2.7). The C-terminal domain has seven β-strands forming a jelly roll barrel motif, and four loops are arranged above the catalytic site. The linking of main core with C-terminal domain through interdomain disulfide bridge necessitates the proteolytic activation of the latent AUS1 to occur at three different sites (Fig. 2.7). This suggests and is well documented by the purification of "partially cleaved" latent enzyme forms from natural source (Molitor et al. 2015a) that proteolytic activation of the enzyme is stepwise and is targeted. The latent AUS1 displayed low activity toward butein and exhibited allosteric activation in presence of *o*-quinones (Molitor et al. 2015a). This indicates that at least a small part of the active site of latent AUS1 is accessible to substrates. Limited or no electron density is found in a loop region of jelly roll motif and in the

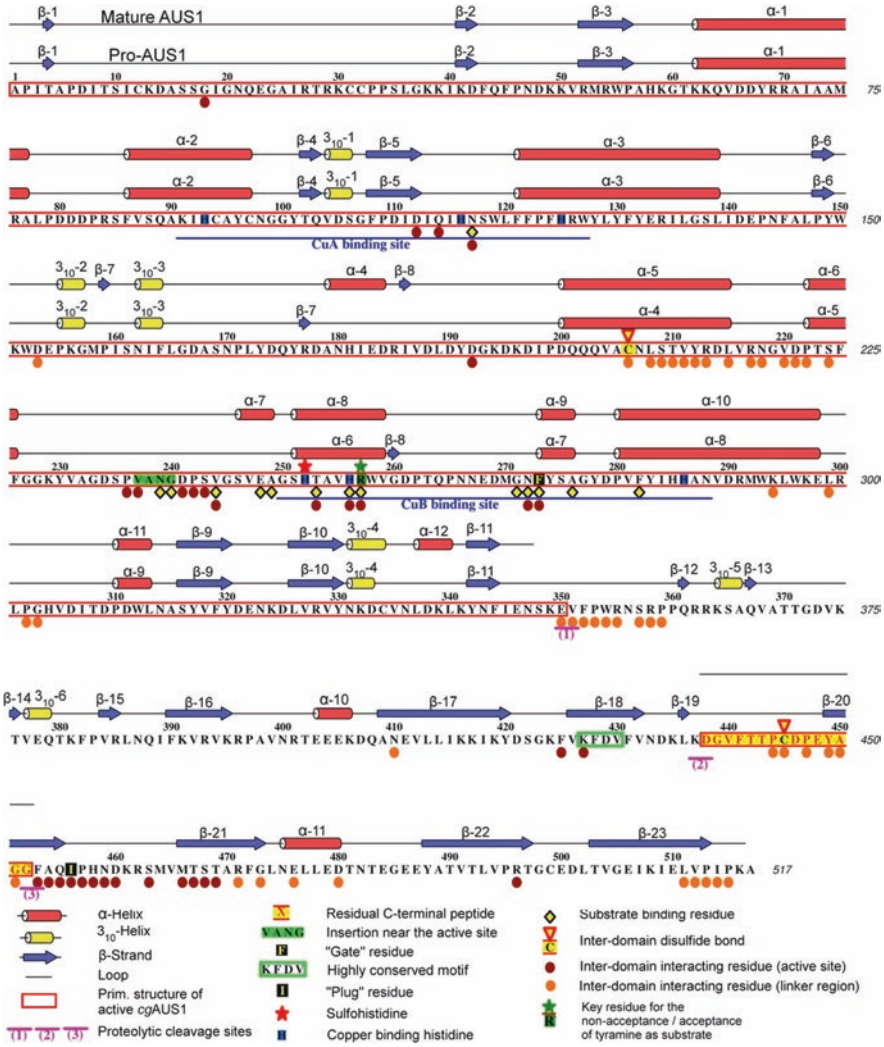

Fig. 2.5c Features of primary and secondary structure of latent and mature AUS1 (Source: Molitor et al. 2016)

loop that carries the insertion demonstrating the possibility of this region being an entrance to the substrates (Fig. 2.6).

The entrance to the active site is blocked by Ile456; this residue functions as a "plug" (Fig. 2.8). The Ile456 is present outside the active site and on top of the Phe273, the gate residue. Hydrophobic interactions of plug residue with gate residue as well as with one CuB binding His252 and Thr253 stabilize the plug residue. An unexpected electron density at CuB binding His252 was displayed in the crystal structure of AUS1 purified from its natural source (Fig. 2.9). The electron density

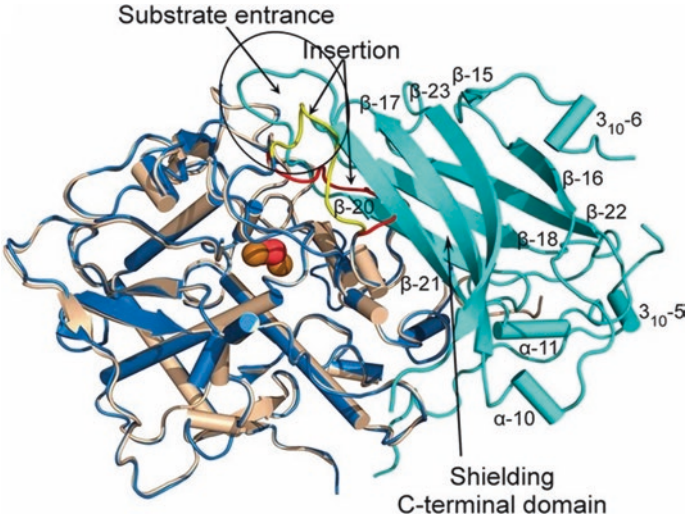

Fig. 2.6 Superposition of active [colored *beige*] and latent [catalytically active domain, *blue*; shielding C-terminal domain, *cyan*] AUS1. The location of the loop carrying the insertion is different in the latent proenzyme [latent, *yellow*; active, *red*] (Source: Molitor et al. 2016)

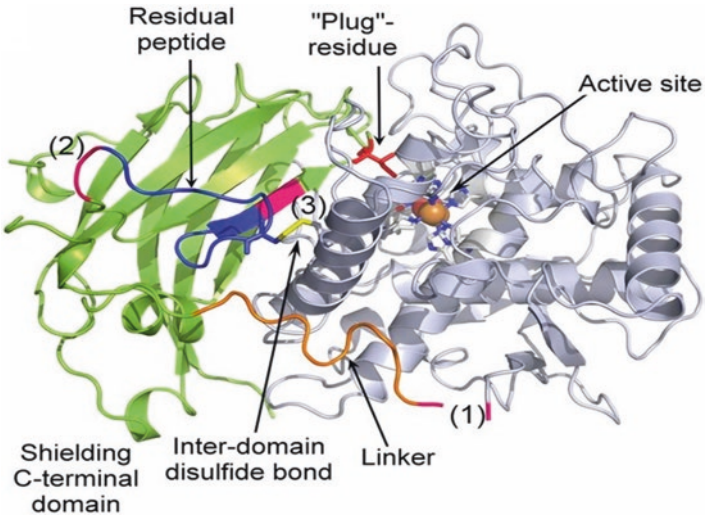

Fig. 2.7 Latent AUS1 structure. The features of the C-terminal domain are three proteolytic cleavage sites [*magenta*, labeled as 1, 2, and 3; linker region [*orange*] connecting the catalytically active [*gray*] and the shielding C-terminal domain [*green*]; residual peptide of the C-terminal domain [*blue*] and the active site shielding Ile456 [plug residue; *red*] (Source: Molitor et al. 2016)

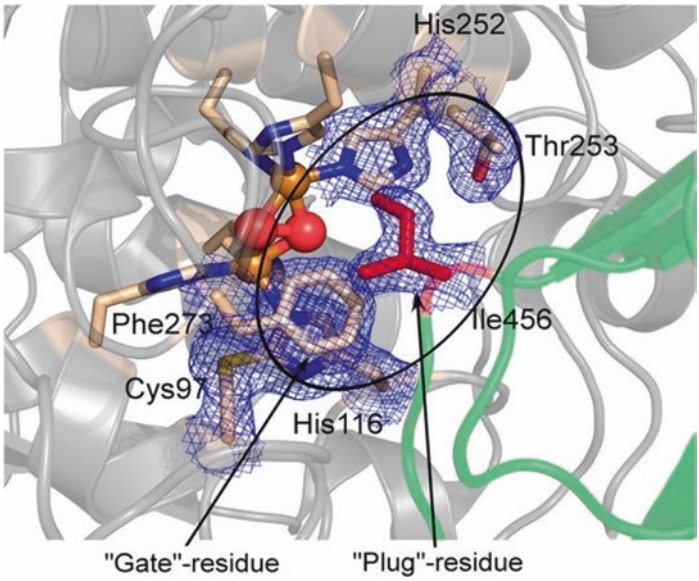

Fig. 2.8 Shielding of the active site by the C-terminal residue Ile456 (Source: Molitor et al. 2016)

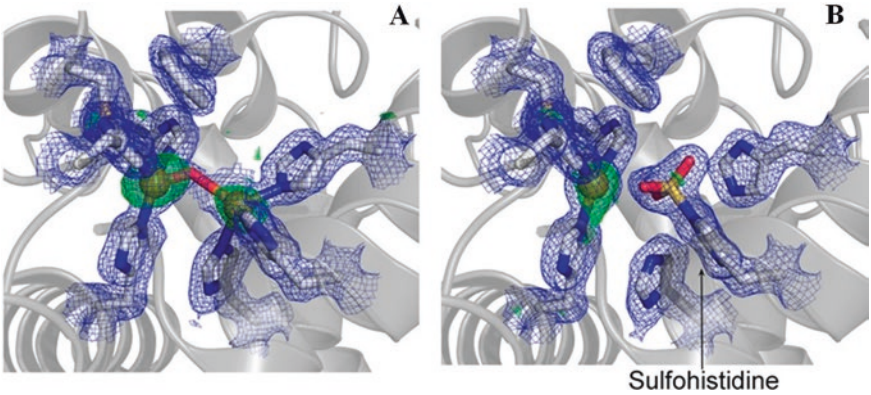

Fig. 2.9 (**a**) Copper-binding site of active AUS1 [occupancy CuB, ~0.55]. For clarity, the sulfohistidine [occupancy, ~0.45] is not shown. (**b**) Inactive AUS1 [occupancy sulfohistidine, 0.9] (Source: Molitor et al. 2016)

observed was more so in the enzyme samples that had increased levels of sulfation or phosphorylation (Molitor et al. 2015b), making it impossible to put a histidine-coordinated copper atom into the density. But the density of a phosphohistidine or sulfohistidine matched very well with this density. Further, sulfation of histidine residue was revealed by mass spectrometry, resulting in the loss of histidine's ability to involve in copper coordination. Sulfation and loss of coordination ability further

lead to loss of CuB atom and ultimately the loss of enzymatic activity of the modified enzyme.

Earlier studies reported that AUS1 displays only diphenolase (oxidation of diphenolic substrates to their corresponding *o*-quinones) and completely lacks monophenolase activity (hydroxylation of monophenolic substrates; Kaintz et al. 2014; Molitor et al. 2015a). But Molitor et al. (2016) have shown that AUS1 exhibited hydroxylase activity toward isoliquiritigenin when the activity assay is modified by addition of ascorbic acid, but in absence of ascorbic acid, the quinoid intermediates and products polymerize and become untraceable by HPLC analysis. Further, sulfuretin, the reaction product, is described to be a suicide substrate for AUS1 (Molitor et al. 2015a). In the absence of a reducing agent, the difference spectra of isoliquiritigenin with AUS1 and *Agaricus bisporus* tyrosinase (*ab*TYR) differed. But, in the presence of a reducing agent, the difference spectra exhibited identical absorption changes indicating that the differences observed were due to complex consecutive reactions and not because of hydroxylation reaction. Additionally, the simulation studies of isoliquiritigenin and tyramine, both monophenols, with *oxy*-form of AUS1 demonstrated the stability of isoliquiritigenin within the cavity of active site and had identical enzyme–substrate interactions as compared to butein and lanceoletin, both diphenols even under Cu–O substrate distance restraint conditions. But tyramine left the cavity of AUS1 within 75 ps due to attractive interaction with Arg257 residue, which demonstrates the lack of monophenolase activity of AUS1 toward tyramine. To understand the role of Arg257 residue, simulation studies involving tyramine with *Juglans regia* tyrosinase (*jr*TYR) and L244R mutant form of *jr*TYR revealed that tyramine remained stable in the active site of *jr*TYR but left the site of mutant L244R immediately. These results suggest a critical role for the residues at this position for accepting or denying tyramine to be a substrate for plant PPOs.

2.2.3 Structure of Walnut (*Juglans regia*) Tyrosinase

The first crystal structure of a plant tyrosinase in its active form was determined for walnut tyrosinase (*jr*TYR; isolated from its leaves) using X-ray crystallography to a resolution of 1.8 Å (Zekiri et al. 2014; Bijelic et al. 2015). The *jr*TYR shares significant structural similarity with plant catechol oxidases from *Ipomoea batatas* (main core identity is ~57 %; Klabunde et al. 1998) and *Vitis vinifera* (64 %; Virador et al. 2010). The active site region of *jr*TYR containing the copper center is also formed by a four-α-helix bundle (α4, α5, α12, α14; Fig. 2.10), and each copper ion is coordinated by histidine residues (Fig. 2.10). Copper A (CuA) is coordinated by His87 (located on the α4 helix), His108 (on a loop), and His117 (α5 helix). Similarly, the CuB is coordinated by His239, His243, and His273, all the three histidines are located on α-helices. Limited flexibility of His108 is possible due to the formation of thioether bond between Cε atom of His108 and sulfur atom of an adjacent Cys91. A solvent molecule, most probably a hydroxide anion, bridges the two copper centers and is equidistant (2.1 Å) from each copper ion. The *jr*TYR structure contains two disulfide bonds (Cys11–Cys26 and Cys25–Cys88), they stabilize the N-terminal

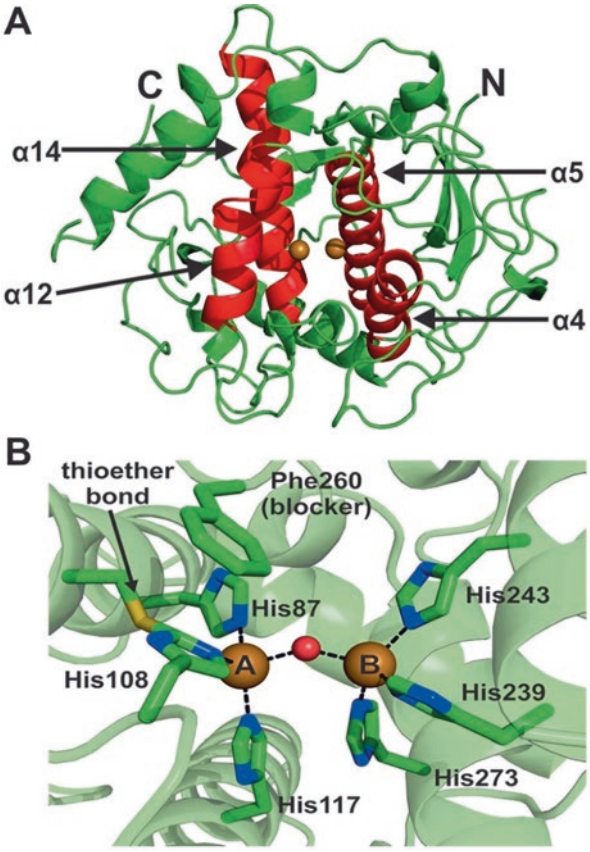

Fig. 2.10 (**a**) The overall structure [*green*] and four α-helical bundles forming the active site [*red*]. (**b**) The active site with the copper-coordinating histidine residues, blocker residue, and thioether bond (*green* C, *blue* N, *yellow* S). The copper atoms are shown as *brown* spheres with the bridging solvent as a small *red* sphere (Source: Bijelic et al. 2005)

loops by anchoring them with main core and are also associated with copper incorporation. The *jr*TYR has the well-conserved tyrosinase CXXC motif (C88- A-Y-C91), shown to be crucial for copper uptake (Fujieda et al. 2013).

A Phe260 is present at the blocker residue position above CuA in *jr*TYR (Fig. 2.10), an interesting feature considering that bulky blocker residues are observed in catechol oxidases (Klabunde et al. 1998; Matoba et al. 2006). Further, the presence of a bulky phenylalanine residue at the CuA site has been assumed to prevent the binding of substrates at CuA, thereby resulting in total lack of monophenolase activity (Sendovski et al. 2011; Olivares et al. 2002; Gerdemann et al. 2002). This prompted researchers to believe that monophenols bind to CuA and diphenols to CuB, but Goldfeder et al. (2013) have shown that monophenols and diphenols bind to CuA by determining the crystal structure of *Bacillus megaterium* tyrosinase (*bm*TYR). Further, it was indicated that both the substrates bind to CuA site in catechol oxidase (Goldfeder et al. 2014). Limited flexibility of CuA site due to the thioether bond coupled with a bulky blocker residue is responsible for the lack of monophenolase activity in catechol oxidases, as both these conditions prevent the substrate rotation which is believed to be required for hydroxylation of

monophenols (Decker et al. 2006a, b; Goldfeder et al. 2014). Interestingly, *jr*TYR having both the conditions (Zekiri et al. 2014) has been shown to have monophenolase activity, indicating undoubtedly that a restricted active site need not be the reason for mono-/diphenolase activity.

A phenylalanine residue is in the blocker residue position in *Ipomoea batatas* and *Vitis vinifera* catechol oxidase (*ib*CO, *vv*Co), but studies have shown the flexibility of phenylalanine residue allowing substrate access to CuA (5, 20). Additionally, CuA site in *jr*TYR is freely accessible (Bijelic et al. 2015); therefore, the term "blocker" for residue above CuA site is confusing and should be reconsidered at least in case of plant polyphenol oxidases. Despite minimal differences among the second shell residues (e.g., Leu244 in *jr*TYR) near the active site entrance in plant PPOs, they could be crucial for the difference in activity. Some of these differences result in unique electrostatic behavior at the corresponding positions suggesting that such interactions are critical during substrate binding to both types of enzymes and may affect tyrosinase activity (Molitor et al. 2015a).

Another interesting feature of *jr*TYR was the presence of difference electron density in the active site probably indicating a path from protein surface to the active site and may originate from influxing solvent molecules (Fig. 2.11a). Since a part of this density overlaps CuA, it is assumed that a substrate could be directed toward CuA following this pathway. Further, superimposition studies involving *jr*TYR with substrate-bound *bm*TYR structures overlapped precisely revealing the compatibility between substrate orientation and density of *jr*TYR and also supporting the pathway (Fig. 2.11b). Kinetic studies revealed that monophenols lacking carboxylate moiety had a faster turnover rate probably due to preferential stabilization of more hydrophobic substrates by Leu244, the second shell residue near the active site. Interestingly, the superimposition studies also revealed that the orientation of bulky Phe260 in *jr*TYR is such that it shows a weak T-shaped π–π interaction with aromatic ring of the substrate. The Phe260 along with CuB coordinating His243 forms a specific gate leading into the active site that can only be accessed by substrates in correct orientation. Further, the aromatic rings of all the superimposed substrates which were between Phe260 and His243 and are stabilized by cation–π interactions with His243 have been demonstrated in bmTYR (Goldfeder et al. 2014).

The proposed binding mechanism (Bijelic et al. 2015) involves the pre-orientation of the substrate at the active site entrance by second shell residues through interactions followed by deprotonation by conserved water molecule. Later, the substrate is oriented by Phe260 and His243 and positioned in such a way that the phenol ring of the substrate is directed toward copper bridging oxo ligands (assuming *oxo* form of enzyme). The solvent molecules then assist in hydroxylation reaction; subsequently, the diphenolic intermediaries undergo oxidation to give *o*-quinones. This "substrate-guiding residue" mechanism does not require either much substrate rotation or active site rearrangement because the substrate is introduced in correct orientation.

Overall, it can be concluded that the determination of crystal structure of plant catechol oxidase and tyrosinase in different states has revealed significant and very crucial information required for understanding the working/reaction mechanism of

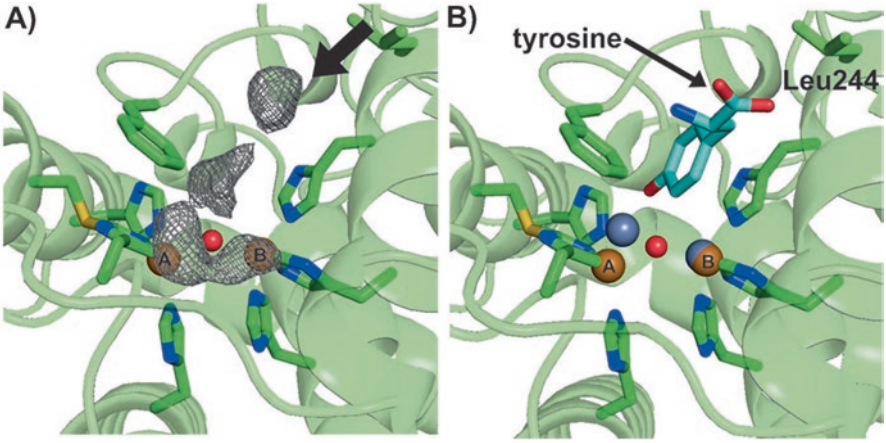

Fig. 2.11 (**a**) Electron density marking the pathway into the active site is illustrated as a *gray* mesh. The *arrow* indicates the direction of the pathway. The two copper ions [*brown* spheres] and bridging solvent molecule [*red* sphere]. (**b**) Superimposition of tyrosine from the *bm*TYR+tyrosine with *jr*TYR. The superimposed zinc ions from *bm*TYR+tyrosinase [*silver* spheres]. The superimposed substrate is indicated to exhibit the same orientation as the path in **a** (Source: Bijelic et al. 2005)

PPOs in plants and also expanding their functional boundaries. This information will be very critical for understanding the already known functions of PPOs and probably also other anticipated roles so far unexplained.

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Physicochemical Properties of Polyphenol Oxidases

3

The presence of multiple molecular forms of polyphenol oxidases (PPOs) in several plant species is very well established (Yoruk and Marshall 2003). In addition to the differences in number, the properties of PPOs within and outside a particular species also differ significantly. Plant PPOs have been extensively studied for their physicochemical properties (Vaughn and Duke 1984; Yoruk and Marshall 2003; Fronk et al. 2015). The PPOs can be distinguished based on the differences in their optimum pH, temperature, and substrate specificity. Here we discuss the impact and importance of these properties on the enzyme activity.

3.1 Impact of pH on PPO Activity

The enzyme activity depends strictly on the pH of the assay or medium (Bisswanger 2014). The activities of majority of enzymes follow a bell-shaped curve, increasing from zero (strongly acidic), reaching a maxima/optima value, and decreasing to zero value in strong alkaline region (Bisswanger 2014). The state of protonation of functional groups of amino acids and cofactors involved in the enzymatic reaction coupled with the native three-dimensional structure of the enzyme affects the enzyme behavior. The protonation could be reversed, but damaging the protein structure is irreversible and could impair the enzyme activity (Bisswanger 2014). Further, protonation could prevent binding of substrates or catalysis of the substrate (Segel 1976; Tipton and Dixon 1983; Whitaker 1994). pH-induced conformational changes in the enzyme could alter the kinetic behavior of PPO (Janovitz-Klapp et al. 1989; Valero and Garcia-Carmona 1992, 1998).

The maximum pH value of the pH-activity curve is the pH optimum, since the enzyme exhibits its highest activity (V_{max}) at this pH and is usually chosen as the standard pH for assay (Bisswanger 2014). Generally, the pH optimum of various plant PPOs is in the range of 4.0–8.0 (Table 3.1). The PPOs of Prunoideae family (almond, apricot, peach, and plum) exhibit a maximum enzyme activity at pH ~5.0 (Fraignier et al. 1995). Fruits like cherry and strawberry exhibit narrow pH optima

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Table 3.1 pH optimum of PPO from different plant sources

Enzyme source	Scientific name	Substrate	pH optimum	References
Almond	*Prunus amygdalus* L.	4-methylcatechol	5.0	Fraignier et al. (1995)
Apple	*Pyrus malus* L.	4-methylcatechol/ chlorogenic acid	3.5–4.5	Marques et al. (1995)
		4-methylcatechol/ catechol	7.0	Oktay et al. (1995)
Apricot	*Prunus armeniaca* L.	4-methylcatechol	5.0–5.5	Fraignier et al. (1995)
Artichoke	*Cynara scolymus* L.	4-methylcatechol/ catechol	6.0	Aydemir (2004)
Avocado	*Persea americana*	4-hydroxyanisole	5.0	Espin et al. (1997)
Banana	*Musa cavendishii*	Catechol	7.0	Ünal (2007)
Cherry	*Laurocerasus officinalis* Roem.	4-methylcatechol	4.5	Fraignier et al. (1995)
		DHPPA[a]	5.0	Colak et al. (2005)
Chestnut	*Castanea henryi*	Catechol	5.0	Xu et al. (2004)
Cocoa	*Theobroma cacao* L.	Catechol	6.8	Lee et al. (1991)
Cucumber	*Cucumis sativus*	Catechol	7.0	Miller et al. (1990)
Dog-rose	*Rosa dumalis* Rechst.	Catechol	8.5	Sakiroglu et al. (1996)
		L-tyrosine	7.0	
		p-cresol	5.0	
Eggplant	*Solanum melongena*	4-methylcatechol/*tert*-butylcatechol	~5.0 to 6.5	Perez-Gilabert and Carmona (2000)
		p-cresol	7.5	
Field bean	*Dolichos lablab*	4-methylcatechol/ catechol	4.0	Paul and Gowda (2000)
		L-DOPA[b]	5.0	
Grape	*Vitis vinifera*	4-methylcatechol	3.5–4.5	Valero et al. (1998)
		Chlorogenic acid/ catechin	5.0	Rapeanu et al. (2006)
Kiwi	*Actinidia chinensis* planch	Catechol	7.3	Park and Luh (1985)
		(+) catechin	8.0	
Lettuce	*Lactuca sativa*	Chlorogenic acid	5.0–8.0	Heimdal et al. (1994)
Longan	*Dimocarpus longan* Lour.	4-methylcatechol	6.5	Jiang (1999)
Loquat	*Eriobotrya japonica* Lindl.	Chlorogenic acid/4-*tert*-catechol	6.5	Selles-Marchart et al. (2006)
Mango	*Mangifera indica*	Catechol/pyrogallol	7.0	Wang et al. (2007)
		4-methylcatechol	5.8	Robinson et al. (1993)

(continued)

Table 3.1 (continued)

Enzyme source	Scientific name	Substrate	pH optimum	References
Medlar	*Mespilus germanica* L.	DHPAA/ epicatechin/L-DOPA	6.5	Dincer et al. (2002)
Mulberry	*Morus alba* L.	Pyrogallol/4-methylcatechol/catechol	7.5	Arslan et al. (2004)
Olive	*Olea europaea*	4-methylcatechol	5.5–7.5	Ben-Shalom et al. (1977)
Peach	*Prunus persica* L.	4-methylcatechol	5.0	Fraignier et al. (1995)
Peppermint	*Mentha piperita*	Catechol	7.0	Kavrayan and Aydemir (2001)
Persimmon	*Diospyros kaki* L.	Catechol/4-methylcatechol	7.5	Özen et al. (2004)
Pineapple	*Ananas comosus*	Catechol	6.0–7.0	Das et al. (1997)
Plum	*Prunus cerasifera* L.	4-methylcatechol	4.0–5.5	Fraignier et al. (1995)
Potato	*Solanum tuberosum*	Chlorogenic acid/ tert-butyl alcohol	4.5–5.0 and 6.0–6.5	Sanchez-Ferrer et al. (1993)
Spinach	*Spinacia oleracea*	Dopamine	8.0	Sheptovitsky and Brudvig (1996)
Strawberry	*Fragaria* sp.	Catechol	5.5	Wesche-Ebeling and Montgomery (1990)
		4-methylcatechol	4.5	
		Catechol	5.0	Dalmadi et al. (2006)
Sunflower	*Helianthus annuus*	Gallic acid	7.9	Raymond et al. (1993)
Tea	*Camellia sinensis* L.	4-methylcatechol	5.0	Gregory and Bendall (1966)
		Pyrogallol	5.7	
Thymus	*Thymus longicaulis* subsp. *chaubardii* var. *chaubardii*	Pyrogallol/4-methylcatechol/catechol	6.5	Dogan and Dogan (2004)
Tomato	*Lycopersicon esculentum*	DOPAC[c]	4.8	Spagna et al. (2005)
Wheat	*Triticum aestivum* L.	4-methylcatechol	5.3 and 6.9	Interesse et al. (1980)
	Triticum durum	Catechol	6.5	Altunkaya and Gökmen (2012)
Yacon root	*Smallanthus sonchifolius*	Caffeic acid/chlorogenic acid/4-methylcatechol	6.6	Neves and Silva (2007)

[a]*DHPPA* 3-(3,4 dihydroxyphenyl) propionic acid
[b]*L-DOPA* L-3,4-dihydroxyphenylalanine
[c]*DOPAC* 3,4-Dihydroxyphenylacetic acid

of ~4.5 with 4-methylcatechol as substrate (Wesche-Ebeling and Montgomery 1990; Fraignier et al. 1995). Apple and grape PPOs show wider optimum pH range of 3.5–4.5 (Valero et al. 1988; Marques et al. 1995). The pH optimum shifts to near-neutral range (7.0) in some apple varieties (Oktay et al. 1995).

Several researchers found acidic pH optimum for apple PPO, but some identified a single pH optima at 4.5–5.0 (Goodenough et al. 1983; Janovitz-Klapp et al. 1989; Zhou et al. 1993). The optimum pH of grape PPO also exhibited wide varietal variation ranging from 3.5 to 7.3 (Cash et al. 1976; Wissemann and Lee 1981; Valero et al. 1988; Lamikanra et al. 1992; Rapeanu et al. 2006). The PPO from pineapple, mango, and longan fruit demonstrated maximum activity at neutral pH with catechol or its derivatives as substrates (Das et al. 1997; Wang et al. 2007; Jiang 1999), whereas kiwifruit's maximum was pH 8.0 with (+) catechin as substrate (Park and Luh 1985; Table 3.1).

Interestingly, the eggplant PPO exhibits wide pH optima depending on the monophenolase or diphenolase activity. In case of the former, there was a wider range of optimum pH (5.0–6.5) with 4-methylcatechol and *tert*-butylcatechol as substrates, while a single pH maxima of 7.5 for the latter with *p*-cresol as substrate was observed (Perez-Gilabert and Carmona 2000). While Dogan et al. (2002) reported a pH optimum of 6.0 and 7.0 for eggplant (aubergine) PPO with 4-methylcatechol and catechol as substrates, respectively, lettuce PPO also exhibited broad pH optima of 5.0–8.0 with catechol, 4-methylcatechol, and pyrogallol as substrates (Heimdal et al. 1994; Dogăn and Salman 2007). On the contrary, spinach exhibited a single pH optimum of 8.0 and showed no or limited activity below pH 6.0 (Sheptovitsky and Brudvig 1996).

The presence of two different ionization states of the enzyme–substrate complex or two acid dissociation constants (pKs) results in two different pH optima in potato (4.5–5.0 and 6.0–6.5; Sanchez-Ferrer et al. 1993) and wheat (5.3 and 6.9; Interesse et al. 1980). The potato and wheat PPO showed both the pH optima with chlorogenic acid/tert-butylcatechol and 4-methylcatechol as substrates (Table 3.1). Two different PPO isoforms in extracts could also show different pH optimum (Yoruk and Marshall 2003). The two sweet potato PPO isoforms exhibited two different pH optima, at 5.4 and 6.7, with chlorogenic acid as substrates (Nozue et al. 1998). Similarly, kiwifruit isoforms exhibited maximum enzyme activity at pH of 6.8 and 7.3 with catechol (Park and Luh 1985). Oba et al. (1992) also reported the presence of two pH optima (5.5 and 6.8) for banana PPO isozymes from bud extracts.

The optimum pH of an enzyme is affected by different factors like temperature, substrate used, extraction methods, and buffer used for determination of pH (Whitaker 1994). The olive PPO exhibited a broad pH optimum range (5.5–7.5) when the enzyme was extracted without any purification process, while a single pH optimum (4.5) was observed when purified from olive acetone powder (Ben-Shalom et al. 1977). The impact of nature of substrate used on the optimum pH has been demonstrated in several plant species including tea, strawberry, dog rose, and bean seeds (Table 3.1). The differences observed could be due to the binding capacity of the phenolic substrates to the catalytic site under acidic and alkaline conditions (Tipton and Dixon 1983; Whitaker 1994). The pH optimum is also subjected to

changes in presence of modulator like SDS. The apple PPO activity was inhibited at acidic pH and activated at pH >5.0 regardless of substrate but in the presence of 3.5-mM SDS (Marques et al. 1995). Sometimes a shift in pH optimum from lower to higher value is observed differing based on substrates as observed in strawberry, peach, *Prunus* fruits, and broad bean PPOs (Yoruk and Marshall 2003). The observed shift could be due to activation of latent PPOs (broad bean; peach) or other causes (apple, strawberry, and *Prunus* fruits; Yoruk and Marshall 2003).

3.2 Effects of Temperature on PPO Activity

Temperature is another vital factor that affects the PPO activity. The temperature dependence of enzyme activity resembles to some extent to pH dependence: increase with rising temperature, reaching a maximum, and decline (Bisswanger 2014). Two counteracting forces are responsible for this behavior: (i) the velocity of chemical reaction rises with temperature at two to three times every 10 °C [true for enzyme reactions], and (ii) the destabilization of thermosensitive three-dimensional structure of enzyme at higher temperature causes denaturation and a decline in reaction velocity at high temperature. Actual temperature coupled with time of exposure limits the progression of denaturation (Bisswanger 2014). The solubility of oxygen, a key substrate necessary for PPO reaction, may be altered due to variable temperature (Whitaker 1994). The effect of temperature on the PPO activity is shown in Table 3.2. The optimum temperature varies significantly for different plant species.

The optimum temperature for maximum PPO activity ranges from 25–35 °C to 25–45 °C in lettuce (Heimdal et al. 1994) and grape (Valero et al. 1988; Rapeanu et al. 2006), respectively, decreasing gradually below and above the range. Several fruit species including mango, apple, banana, and loquat exhibited a temperature optimum of 30 °C (Table 3.2). While cocoa and sunflower show 45 °C as their pH optima, cucumber, sunflower, and cherry exhibit relatively higher temperature optima of 50 °C compared to other plant species (Table 3.2).

Strawberry PPO shows two contrasting pH optima depending on substrates used, 25 °C with catechol (Dalmadi et al. 2006) and 50 °C with pyrocatechol (Serradell et al. 2000). Similarly, dog rose PPO exhibits lower temperature optima with diphenols (catechol; 25 °C) and triphenols (pyrogallol; 15 °C) and higher optima with monophenolic substrates like tyrosine (65 °C) and *p*-cresol (60 °C; Sakiroglu et al. 1996).

The temperature inactivation of majority of plant PPOs has been shown to follow first-order kinetics (Wissemann and Lee 1981; Lee et al. 1983; Wesche-Ebeling and Montgomery 1990; Robinson et al. 1993; Yemenicioglu et al. 1997). But thermal inactivation of sunflower seed PPO followed first-order kinetics at 65 °C but not at higher temperatures of 80 °C and 100 °C (Raymond et al. 1993). Interestingly, the thermal stability was shown to be cultivar dependent in apple; PPO in Amasya cultivar was less heat stable compared to PPO from Starking Delicious apples (Yemenicioglu et al. 1997). Further, differential heat stability of multiple PPO forms of peach was observed (Wong et al. 1971). Exposure time to particular temperature is also very critical for enzyme inactivation and varies significantly among different

Table 3.2 Optimum temperature of PPO from different plant sources

Enzyme source	Scientific name	Substrate	Optimum temperature (°C)	References
Apple	*Pyrus malus* L.	4-methylcatechol/ catechol	15	Marques et al. (1995)
		Catechol	30	Zhou et al. (1993)
Artichoke	*Cynara scolymus* L.	4-methylcatechol/ catechol	25	Aydemir (2004)
Banana	*Musa cavendishii*	Catechol/dopamine	30	Ünal (2007) and Yang et al. (2000)
Cherry	*Laurocerasus officinalis* Roem.	DHPPA[a]	50	Colak et al. (2005)
Chestnut	*Castanea henryi*	Catechol	40	Xu et al. (2004)
Cocoa	*Theobroma cacao* L.	Catechol	45	Lee et al. (1991)
Cucumber	*Cucumis sativus*	Catechol	50	Miller et al. (1990)
Dog rose	*Rosa dumalis* Rechst.	Catechol	25	Sakiroglu et al. (1996)
		L-tyrosine	65	
		p-cresol	60	
		Pyrogallol	15	
Grape	*Vitis vinifera*	4-methylcatechol	25–45	Valero et al. (1998)
		Chlorogenic acid/ catechin	25	Rapeanu et al. (2006)
Lettuce	*Lactuca sativa*	Chlorogenic acid	25–35	Heimdal et al. (1994)
Longan	*Dimocarpus longan* Lour.	4-methylcatechol	35	Jiang (1999)
Loquat	*Eriobotrya japonica* Lindl.	Chlorogenic acid/4-*tert*-catechol	30	Selles-Marchart et al. (2006)
Mango	*Mangifera indica*	Catechol/pyrogallol	30	Wang et al. (2007)
		4-methylcatechol	30	Robinson et al. (1993)
Medlar	*Mespilus germanica* L.	DHPAA[a]/ epicatechin/L-DOPA	35	Dincer et al. (2002)
Mulberry	*Morus alba* L.	Pyrogallol/4-methylcatechol/ catechol	20	Arslan et al. (2004)
Peppermint	*Mentha piperita*	Catechol	30	Kavrayan and Aydemir (2001)
Persimmon	*Diospyros kaki* L.	Catechol/4-methylcatechol	20–40	Özen et al. (2004)
Potato	*Solanum tuberosum*	Catechol	40	Cho and Ahn (1999)

(continued)

Table 3.2 (continued)

Enzyme source	Scientific name	Substrate	Optimum temperature (°C)	References
Strawberry	*Fragaria* sp.	Catechol	25	Dalmadi et al. (2006)
		Pyrocatechol	50	Serradell et al. (2000)
Sunflower	*Helianthus annuus*	Gallic acid	45	Raymond et al. (1993)
Thymus	*Thymus longicaulis* subsp. *chaubardii* var. *chaubardii*	Pyrogallol/4-methylcatechol/ catechol	35	Dogan and Dogan (2004)
Tomato	*Lycopersicon esculentum*	DOPAC[b]	40	Spagna et al. (2005)
Wheat	*Triticum durum*	Catechol	40	Altunkaya and Gökmen (2012)
Yacon root	*Smallanthus sonchifolius*	Caffeic acid/ chlorogenic acid/4-methylcatechol	30	Neves and Silva (2007)

[a]*DHPPA* 3-(3,4 dihydroxyphenyl) propionic acid
[b]*DOPAC* 3,4-Dihydroxyphenylacetic acid

plant sources. Grape PPO showed ~50 % reduction in enzyme activity at 65 °C after 20 min, while complete enzyme inactivation was achieved at 75 °C for 15 min (Valero et al. 1988). The apple PPO has a half-life of 30 min at 60 °C (Zhou et al. 1993). Lettuce PPO is relatively heat stable with no loss of activity up to 70 °C for 5 min, while no activity was detected at 90 °C for 5 min. The phenolic substrate could also impact the thermal stability of the enzyme (Park and Luh 1985; Wesche-Ebeling and Montgomery 1990). The PPO activity of kiwifruit extracts toward catechol was less heat stable compared to (+) catechin (Park and Luh 1985). Sucrose and salts present in the reaction extract could protect the enzyme against thermal denaturation (Lourenco et al. 1992).

3.3 Substrate Specificity of PPOs

Different phenolic compounds are the principal substrates of plant PPOs. Several compounds that serve as substrates to PPOs are listed in Table 3.3. The PPO activity of different plant species shows distinct variation for different substrates based upon the plant source (Table 3.3). There is significant variability for the types and concentrations of natural phenolic substrates among the different plant species. Catechin is the major phenolic substrate in grapes (Jaworski and Lee 1987) and tea (Ullah 1991); chlorogenic acid is key substrate in apple (Murata et al. 1995), potato

Table 3.3 Relative substrate specificity of PPOs

Substrates	Percent activity					
	Apple[a]	Field bean[b]	Grape[c]	Peach[d]	Sunflower seeds[e]	Strawberry[f]
Monophenols						
Tyrosine	3	0	0	0	0	0
p-cresol	–	0	0	0	0	–
p-coumaric acid	–	–	–	0	–	0
Diphenols						
Catechol	100	100	5.9	100	–	9
4-methylcatechol	181	140	74	103	–	80
Chlorogenic acid	102	0	51	–	32.3	11
L-DOPA	–	22.6	5.4	23	–	–
Catechin	54	0	21	539		100
Caffeic acid	–	0	100	7	87.3	13
Triphenols						
Gallic acid	–	0	0	5	100	–
Pyrogallol	38	24	0	182	100	62

[a]Zhou et al. (1993)
[b]Paul and Gowda (2000)
[c]Lee et al. (1983)
[d]Flurkey and Jen (1980)
[e]Raymond et al. (1993)
[f]Wesche-Ebeling and Montgomery (1990)

(Sanchez-Ferrer et al. 1993), sunflower (Raymond et al. 1993), sweet potato (Lourenco et al. 1992), and eggplant (Stommel and Whitaker 2003); and ferulic acid is abundant in wheat (Laddomada et al. 2016). Further, catechin, epicatechin, and caffeic acid derivatives are common substrates of several fruit PPOs (Macheix et al. 1990). The oxidation of dopamine by endogenous PPO was shown to cause enzymatic browning in banana pulp (Yang et al. 2000; Sojo et al. 1998). Studies indicate the degree of darkening is dependent upon the type of substrate. The monomeric catechins and dimeric procyanidins cause more intense browning compared to other phenolics in grape (Lee and Jaworski 1988). Similarly, catechin was shown to be mostly responsible for browning in apples (Murata et al. 1995). In eggplant, 4-methylcatechol has been reported to be a good substrate in comparison to gallic acid or pyrogallol (Mishra et al. 2012).

PPOs exhibit higher activity with those substrates that show high affinity or preference toward the enzyme. The catalytic activity of the enzyme is influenced significantly by the nature of the side chain, number of hydroxyl groups, and their position on the benzene ring of the phenolic substrate (Harel et al. 1964; Macheix et al. 1990; Park and Luh 1985). PPOs from some plant species like grape, field bean seed, sunflower seed, and strawberry have exhibited only diphenolase activity (Yoruk and Marshall 2003). The apple PPO lacked monophenolase activity (Choi et al. 1987; Walker 1964) or, when present, was very low compared to the diphenolase activity. The monophenolic substrates were phloretin/phloridzin (Challice 1973), *p*-cresol/*p*-chlorophenol (Harel et al. 1964), and *p*-coumaric acid (Goodenough et al. 1983).

Interestingly, tyrosine was never shown to be the substrate of apple PPO (Challice 1973; Choi et al. 1987; Goodenough et al. 1983; Harel et al. 1964; Nakabayashi 1954; Walker 1964). Further, the substrate specificity of PPOs also varies considerably with species and cultivars. The PPO activity in Koshu grapes is highest with chlorogenic acid followed by caffeic acid and *d*-catechin (Nakamura et al. 1983), but PPO from Concord variety oxidizes catechol faster than caffeic acid (Cash et al. 1976). Similarly, caffeic is the best substrate for De Chaunac grape variety PPO (Lee et al. 1983). Additionally, the PPO isozymes in a particular tissue could also exhibit variations in substrate specificities and their relative activities toward the substrates (Bouchilloux et al. 1963; Harel et al. 1964, 1965; Constantinides and Bedford 1967; Wong et al. 1971; Park and Luh 1985; Thomas and Janave 1986; Oba et al. 1992). For example, the two banana isoforms exhibit maximum activity toward dopamine but showed variations in their activities toward chlorogenic acid and L-DOPA and (+) catechin (Oba et al. 1992).

3.4 Multiplicity of Plant PPOs

Multiplicity of PPOs has been observed in several plant species as described earlier (see chapter on "Distribution, Localization, and Structure of Plant Polyphenol Oxidases"). These multiple forms differ in their physical, chemical, and enzymatic properties like temperature, pH optima, and substrate specificity as will be discussed. These distinguishable multiple forms of PPOs could be of significant physiological importance in vivo (Yoruk and Marshall 2003). The multigene families of PPOs have been widely studied in members of *Solanaceae* (tomato, potato, and eggplant), *Poaceae* (wheat, barley and rice), and few other crop species in which the PPOs have directly impacted economically and agronomically important traits (Taranto et al. 2017). Despite several reports regarding the multiplicity of PPOs, ambiguity exists with regard to the exact number of PPO forms in tissues of interest in certain species. Lineage-specific expansion/duplication and gene loss are two important reasons for the extreme variability of size and structure observed in land plant PPOs (Tran et al. 2012). Early works in apple extract identified four active PPO forms (Harel et al. 1965; Harel and Mayer 1968), but subsequent studies reported only two forms (Marques et al. 1995; Zhou et al. 1993). On the contrary, in grapes, eight bands were stained for PPO activity (Harel et al. 1973; Interesse et al. 1984; Sanchez-Ferrer et al. 1989a) against single PPO form reported by Goodenough et al. (1983) and Murata et al. (1995).

The variability of PPO forms could be responsible for the spatial, temporal, and developmental stage or varietal differences (Yoruk and Marshall 2003). The multiple forms of PPOs are sometimes regarded as artifacts with different physical and chemical properties compared to the native enzyme. Such molecular forms could result from association–dissociation phenomena (Harel and Mayer 1968; Goldbeck and Cammarata 1981; Das et al. 1997), interconversion between PPO forms due to aging or purification (Harel and Mayer 1968; Goldbeck and Cammarata 1981), and enrichment of the enzyme or other treatments like GA3, a phytohormone (Saluja

and Sachar 1982). Artifacts could also form due to the attachment of carbohydrates (Flurkey and Jen 1980; Wesche-Ebeling and Montgomery 1990; Raffert and Flurkey 1995) or phenolic oxidation products like quinones (Interesse et al. 1984; Kowalski et al. 1992) to native enzyme in vivo or during extraction/purification procedures (Yoruk and Marshall 2003). The covalent attachment of glycosides to PPO during isolation could result in modification of protein affecting its physicochemical properties including solubility and stability (Flurkey and Jen 1980; Wesche-Ebeling and Montgomery 1990; Cary et al. 1992; Robinson and Dry 1992; Chevalier et al. 1999). Further, the modified enzyme (due to carbohydrate or glycosidic attachment) could be subjected to nonspecific degradation by hydrolytic enzymes resulting in multiple forms (Harel et al. 1973). Glycosylation could be responsible for the observed differences in the molecular weight determined from PPO cDNA and those under fully denaturing conditions (Cary et al. 1992).

Proteases could also be responsible for the generation of PPO artifacts (Flurkey and Jen 1980; Marques et al. 1994; Shin et al. 1997). Therefore, protease inhibitors are used during the extraction and purification procedure to avoid the generation of multiple forms by proteolysis with endogenous proteases. Despite using protease inhibitors, multiple forms were observed in mung bean leaf (Shin et al. 1997) and sweet potato (Nozue et al. 1998). Further, the multiple forms generated due to proteolysis in apple had different biochemical properties like pH (Marques et al. 1995). Activation of latent PPO forms by endogenous proteases or through other means may also contribute to the multiple forms probably differing in their physicochemical properties like pH and molecular weight (Jimenez and Garcia-Carmona 1996; Espin and Wichers 1999). Detergents or denaturing agents (Moore and Flurkey 1990), rigorous isolation procedures (Guillard and Richard-Forget 1997; Yoruk and Marshall 2003), prolonged storage, or exposure to acid pH/urea (Lerner et al. 1972; Harel et al. 1973) could account for multiplicity of the enzyme. The differences in PPO processing and translocation of the products into the plastid may also result in multiple forms (Sommer et al. 1994).

As mentioned above, many of these reports of multiplicity of PPO genes do not add any new details about PPOs; therefore, only some studies which identified genes coding for PPOs are mentioned. Tissue-specific and differential expression studies of multigene PPOs have been undertaken in some crops including wheat (Jukanti et al. 2006), potato (Thygesen et al. 1995), tomato (Thipyapong et al. 1997), eggplant (Shetty et al. 2011), apple (Boss et al. 1995; Kim et al. 2001), and poplar (Tran and Constabel 2011). Results presented in these studies provide important insights into the processing, expression, and regulation of plant PPOs. Interestingly, Tran et al. (2012) have identified a large PPO gene family (13 genes) in a bryophyte moss, *Physcomitrella patens*. Gene knockout studies by Richter et al. (2012) demonstrated the involvement of *P. patens* PPO in plant development, and it was suggested that other PPOs could be responsible for other functions. Most importantly, this study addressed a major lacuna of scarcity of functional knowledge of PPOs outside the seed plant group. Another interesting find was identification of multigene PPO family of 11 members in *Selaginella moellendorffii*, a lycopod with one of the smallest genome among plants (Banks 2009).

3.5 Latency and Activation of PPOs

As described above, the plant PPOs are assumed to be mostly membrane bound (Tolbert 1973; Goldbeck and Cammarata 1981; Lieberei et al. 1981; Meyer and Biehl 1981; Vaughn and Duke 1984; Sanchez-Ferrer et al. 1989b, 1990; Amorim and Melo 1991; Murata et al. 1997). The latent forms of PPO present on the thylakoid membranes are not involved in the oxidation of phenolic substrates, unless they are activated either due to injury, stress, or senescence (Yoruk and Marshall 2003). Binding of the enzyme to the plastidic membrane alone may not result in latency as demonstrated in several plant species including peach (Laveda et al. 2000, 2001), sago palm (Onsa et al. 2000), broad bean (Kenten 1957; Moore and Flurkey 1990; Robinson and Dry 1992; Jimenez and Garcia-Carmona 1996), and pear (Guillard and Richard-Forget 1997).

The latency has been a subject of intense investigation over the years, and several treatments or activating agents have been reported that free the enzyme from its latent state as shown in Table 3.4. Several treatments as outlined in Table 3.4 are proposed for enzyme activation, but the mechanism by which PPOs are activated in vitro is still not clearly elucidated. Proteases such as trypsin and pancreatin have been shown to activate PPOs and increase its solubilization (Goldbeck and Cammarata 1981; Sanchez-Ferrer et al. 1989a; Harel et al. 1973), but others like chymotrypsin, papain, and carboxypeptidase have had limited impact (Harel et al. 1973). Additionally, trypsin treatment has also been shown to convert inactive pro-PPO forms to active form in *Daucus carota* cell cultures and to interconvert isozyme forms (Mayer and Harel 1979). Interestingly, wheat PPO activation through trypsin is believed to be due to nonspecific protein–protein interaction and not through proteolytic action of the protease (Saluja and Sachar 1982).

Generally, it is believed that the enzyme activation by endogenous proteases is done by cleaving a specific fragment, i.e., proteolytic digestion of the latent enzyme

Table 3.4 Different treatments/activating agents for activation of PPOs

S. no	Activating agent	References
1	Detergents	Angleton and Flurkey (1984), Sanchez-Ferrer et al. (1989a, 1993), Moore and Flurkey (1990), Jimenez and Garcia-Carmona (1996), Sojo et al. (1998), Espin and Wichers (1999), Laveda et al. (2000), Jukanti et al. (2003), and Sellés-Marchart et al. (2007)
2	Proteases	Tolbert (1973), Saluja and Sachar (1982), King and Flurkey (1987), Robinson and Dry (1992), Laveda et al. (2001), and Jukanti et al. (2006)
3	Mild heat	Sheptovitsky and Brudvig (1996)
4	Sonication	Leeuwen and Wichers (1999)
5	Denaturants	Robb et al. (1964), Swain et al. (1966), Lerner et al. (1972), and Saluja and Sachar (1982)
6	Alcohols	Guillard and Richard-Forget (1997) and Onsa et al. (2000)
7	Fatty acids	Goldbeck and Cammarata (1981), Guillard and Richard-Forget (1997), and Onsa et al. (2000)
8	Frost and aging	Lieberei and Biehl (1978) and Meyer and Biehl (1980)

(Rathjen and Robinson 1992; Robinson and Dry 1992; Laveda et al. 2001). In vitro proteolytic cleavage of latent broad bean PPO of 60 kDa resulted in a 42-kDa active PPO form, probably due to the loss of protease-sensitive sites at the carboxy-terminal of the enzyme (Robinson and Dry 1992). In vitro activation of wheat kernel PPO through limited proteolysis has also been demonstrated (Jukanti et al. 2006). Interestingly, in vivo carboxy-terminal processing has been shown to be a prerequisite for the latent grape PPO activation (precursor – 60 kDa; active form, 40 kDa) in the chloroplast (Rathjen and Robinson 1992; Dry and Robinson 1994). The requirement of in vivo processing of carboxy-terminal fragment in grape was further supported by (1) a positive correlation between 40-kDa protein quantity and PPO activity and (2) presence of latent 60-kDa form in young grape berries only and its accumulation in mutant grape having negligible PPO activity (Rathjen and Robinson 1992). Structure modelling studies of plant PPOs against a molluscan hemocyanin revealed that plant PPO activation is correlated to the removal of a carboxy-terminal peptide fragment shielding the active site from substrate access (Gerdemann et al. 2002a).

Detergents like sodium dodecyl sulfate (SDS) have been widely used to activate the latent PPO from several plant sources (Yoruk and Marshall 2003). Generally, the SDS treatment results in loss of biological activity of several enzymes owing to severe alteration of tertiary and quaternary structure of the proteins, but the presence of disulfide bonds/bridges in PPOs probably strengthens their structure (Fraignier et al. 1995; Marques et al. 1995; Mari et al. 1998). The activation of latent PPO by SDS follows a sigmoidal pattern with increasing SDS concentrations, but inhibition of PPO activity is observed beyond a certain micelle concentration for the detergent (Moore and Flurkey 1990; Jukanti et al. 2003). There are conflicting reports regarding the dependency of degree of activation on the different substrates used (Sanchez-Ferrer et al. 1993; Jimenez and Garcia-Carmona 1996). Further, enzyme activation by SDS is maximum, above pH 4.0, below which there is neither activation nor inhibition (Moore and Flurkey 1990; Jimenez and Garcia-Carmona 1996; Escribano et al. 1997; Laveda et al. 2000) probably due to the presence of a specific pH-sensitive SDS binding site (Jimenez and Garcia-Carmona 1996). But activation at higher pH values is due to the combined effect of SDS and pH (Jimenez and Garcia-Carmona 1996). The SDS-mediated activation of PPO is probably due to limited conformational changes affected in the enzyme structure (Robb et al. 1964; Swain et al. 1966; Moore and Flurkey 1990). It is speculated that access to the active site is controlled by regulatory fragment which is acted upon by SDS in response to pH or cleaved by proteases (Marques et al. 1995; Jimenez and Garcia-Carmona 1996). It is interesting to note that activation procedure/method plays an important role in the enzyme activity in response to pH. The SDS-activated PPO shows limited or no enzyme activity at pH <4.0, whereas the protease-activated PPO is extremely active at acidic pH (Jimenez and Garcia-Carmona 1996). Activation of PPO by other agents like fatty acids, denaturants, and alcohols could also be due to conformational changes brought about in the PPO structure by these agents (Yoruk and Marshall 2003). Soler-Rivas et al. (1997) reported that pathogen attack could also

trigger PPO activation, indicating the presence of in vivo activation mechanism in response to infection.

3.6 Regulation of Polyphenol Oxidases

Generally, it is agreed that PPO activity is much higher in young tissues (e.g., unripe fruits and young leaves) than in mature ones (Ben-Shalom et al. 1977; Vamos-Vigyazo 1981; Lanker et al. 1987; Felton et al. 1989; Mayer and Harel 1991; Nicolas et al. 1994; Steffens et al. 1994; Murata et al. 1995; Serradell et al. 2000) probably indicating their role in plant defense. The decrease in activity could be due to several reasons including proteolytic degradation, conformational changes, and reduced concentration of enzyme activators or substrates (Barrett et al. 1991; Murata et al. 1995; Laveda et al. 2000). But several studies indicate a transcriptional control of enzyme activity as expression of PPO transcripts decreases with the development progression (Rathjen and Robinson 1992; Shahar et al. 1992; Hunt et al. 1993; Dry and Robinson 1994; Boss et al. 1995; Thygesen et al. 1995). Interestingly, in potato, a moderate level of transcript expression is observed throughout tuber development (Thygesen et al. 1995) probably due to development and tissue-specific expression of different PPO genes. In some cases, PPOs synthesized at early developmental stages are maintained steadily throughout different growth stages in vivo (Hunt et al. 1993; Dry and Robinson 1994; Chevalier et al. 1999; Gooding et al. 2001).

At times substantial PPO activity is observed with no PPO mRNA transcripts at late stages suggesting both translational and posttranslational controls regulating PPO activity as observed in apricot (Chevalier et al. 1999) and wheat (Jukanti et al. 2006). The activation of pre-existing PPOs due to loss of membrane integrity during ripening (as in banana) or senescence (as in spinach) could cause significant PPO activity with limited gene expression (Gooding et al. 2001; Meyer and Biehl 1980, 1981; Lieberei et al. 1981). The pre-existing enzymes may under certain conditions be activated by limited conformation changes (Lerner et al. 1972; Goldbeck and Cammarata 1981; Valero and Garcia-Carmona 1992; Yoruk and Marshall 2003), dissociation of PPO-bound inhibitor complex (Kenten 1957; Lieberei and Biehl 1978; Interesse et al. 1980, 1984; Angleton and Flurkey 1984; Lamikanra et al. 1992), or partial proteolysis (King and Flurkey 1987; Sanchez-Ferrer et al. 1990; Robinson and Dry 1992; Dry and Robinson 1994; Jimenez and Garcia-Carmona 1996). Marques et al. (1995) suggested a posttranslational regulation of inactive PPO forms in apple.

Two major groups of posttranslational protein modification processes are mostly observed: (i) proteolytic processes that are mostly cleavage of specific peptide bonds, resulting in the removal of some part of the polypeptide fragment, and (ii) processes that involve modification of the amino acid side chains – these processes generally do not interfere with the polypeptide backbone. These modifications of protein have varied chemical nature and different functions (Knorre et al. 2009). In this section we will discuss the second group of posttranslational modifications only as the first group has already been discussed. The three important amino acid side

chain modification processes reported in plant PPOs are phosphorylation, glycosylation, and myristoylation.

The addition of a phosphate group to the amino acid side chain is referred to as phosphorylation. Protein kinases (phosphotransferases, EC 2.7.) are enzymes that can add phosphate groups to the side chains of amino acids in different proteins (Hubbard 2009; Hubbard and Miller 2007; Ubersax and Ferrell 2007; Beene and Scott 2007; Alemany et al. 2007; Saltiel and Pessin 2002; Maures et al. 2007; Patwardhan and Miller 2007). The donor of a phosphate group in such reactions is the γ-phosphate ATP group. Based on the type of amino acids to which a phosphate is added, the kinases are grouped into tyrosine kinases [E.C. 2.7.10.2] and serine/threonine kinases [E.C. 2.7.11.1] (Hubbard 2009). Histidine kinases are also often reported in plants, bacteria, and fungi. The histidine kinases function in a two-step signal transduction system (Lieser et al. 2005) involving the transfer of inorganic phosphate residue attached to a histidine in the enzyme onto an aspartate residue in the target protein followed by phosphorylation of the aspartate residue (Lieser et al. 2005).

An undetermined amino acid residue of the C-terminal fragment of *Spinacia oleracea* PPO (SoPPO) was reported to be phosphorylated (Race et al. 1995). Marusek et al. (2006) using different bioinformatics tools identified a serine in the linker region that is predicted to be phosphorylated in all the 11 analyzed plant PPOs. As the linker region is exposed on the surface, the potential phosphorylated serine residue is assumed to be accessible to kinases. The location of the serine residue is also in accordance with the report of phosphorylation of a residue located in the C-terminal region of SoPPO (Race et al. 1995). Further, it has been suggested that the phosphorylation sites are mostly located within the disordered regions as evidenced by the enrichment of disorder-promoting residues around the phosphorylation sites (Iakoucheva et al. 2004). Several potential phosphorylation sites have been predicted in the deduced amino acid sequence of "Grenache" grape leaf PPO (Marusek et al. 2006). Though potential phosphorylation sites were predicted, no electron density corresponding to covalent modification of any side chains was observed (Virador et al. 2010). Interestingly, Sokolenko et al. (1995) indicated that a transmembrane-spanning C-terminus of PPO could be phosphorylated.

Glycosylation of proteins plays a very important role in the eukaryotic cell functioning (Knorre et al. 2009). Glycosylation involves the modification of the functional groups of asparagine residue side chains (*N*-glycosylation) and the OH groups of serine and threonine residues (O-glycosylation). The carboxyamide nitrogen atom of an asparagine residue in the context Asn-XSer/Thr in a protein undergoes *N*-glycosylation. N-glycoside formation begins in the endoplasmic reticulum. The oligosaccharyltransferase enzyme [E.C. 2.4.1.119] transfers a branched tetradecasaccharide fragment ($Glc_3Man_9(GlcNAc)_2$), and this comes from dolichol pyrophosphate (the carbohydrate donor molecule) onto the target protein. Different glycosidases and glycosyl transferases assure the wide variety of glycoproteins by processing the protein-bound tetradecasaccharide residues.

Though *N*-glycosylation at different sites is shown to be essential for proper folding and activity of mammalian tyrosinase (Branza-Nichita et al. 2000; Olivares

et al. 2003), there is very limited evidence suggesting the same in plant and fungal PPOs (Robb et al. 1965, Balasingham and Ferdinand 1970; Stelzig et al. 1972; Flurkey and Jen 1980; Zawistowski et al. 1988; Wesche-Ebeling and Montgomery 1990; Gerritsen et al. 1994; Raffert and Flurkey 1995; Kwon and Kim 1996). However, even these studies do not provide any definitive proof of glycosylation in any of the plant and fungal PPOs. An asparagine located in the N-terminal domain of the *Octopus dofleini* hemocyanin (OdHC; functional unit G) is glycosylated (Cuff et al. 1998). *N*-linked glycosylation in plant and fungal PPOs is not possible since they do not have an asparagine at this position. Despite the lack of concrete evidence, several potential *N*-glycosylation sites were identified based on deduced amino acid sequence of leaf PPO from "Grenache" variety of grapes (Virador et al. 2010). Marusek et al. (2006) predicted possible *N*-β-GlcNAc glycosylation of N-terminally located asparagine in some plant PPOs. Though the asparagine was conserved in all but one plant PPO (*Spinacia oleracea* PPO) analyzed, glycosylation was predicted in only six PPOs (*Lycopersicum esculentum* PPO, LesPPO; *Solanum tuberosum* PPO, StPPO; *Trifolium pratense* PPO, TpPPO; *Vicia faba* PPO, VfPPO; *Nicotiana tabacum* PPO, NtPPO; and *Triticum aestivum* PPO, TaPPO). Similarly, *O*-β-GlcNAc glycosylation of serine or threonine in the N-terminal domain was also predicted to be glycosylated in five plant PPOs (*Ipomoea batatas* catechol oxidase, IbCO; *Lycopersicum esculentum* PPO, LesPPO; *Solanum tuberosum* PPO, StPPO; *Vitis vinifera* PPO, VvPPO; and *Oryza sativa* subsp. *japonica* PPO, OsPPO). Further, several eggplant (*Solanum melongena* L.) PPOs were predicted to have N-glycosylation sites (Jukanti and Bhatt 2015).

The most well-known modifications by addition of fatty acid residues are (1) myristoylation, addition of a CH3– (CH2)12–CO– residue to the amino group of an N-terminal glycine (Walsh et al. 2005; Desmeules et al. 2007; Farazi et al. 2001), and (2) palmitoylation – addition of a CH3–(CH2)14–CO– residue at the SH group of a cysteine residue (Walsh et al. 2005; Smotrys and Linder 2004; Tanimura et al. 2006). The appropriate acyl-coenzyme A is responsible for acylation in both processes; the acyl-coenzyme A is produced during oxidative decay of longer fatty acids. Though there is some speculation about fatty acid-based modification of plant PPOs, there is not much reported about myristoylation of plant PPOs. The occurrence of at least six potential sites of myristoylation was reported in "Grenache" variety of grapes (Virador et al. 2010); this suggests membrane association (Martin and Busconi 2000).

The PPO activity is shown to be induced locally at the site of infection or wounding (Boss et al. 1995; Ray and Hammerschmidt 1998) or systemically probably to protect against future attacks (Thipyapong et al. 1995) or both (Bashan et al. 1987; Constabel et al. 1995; Thipyapong and Steffens 1997). The wound-inducible PPO activity and PPO mRNAs were reported in several plant species including apple, tomato, potato, and tobacco (Boss et al. 1995; Constabel et al. 1995; Thipyapong et al. 1995; Thipyapong and Steffens 1997; Constabel and Ryan 1998). The expression of PPO genes is regulated by wound-inducible octadecanoid signaling pathway (Constabel et al. 1995). Systemin and methyl jasmonate, products of signaling pathway, induce the synthesis of PPO proteins and thereby promote plant defense in

wounded tomato plants. Among the seven tomato PPO genes, only one has been shown to be differentially regulated in response to infections by bacteria/fungus, artificial injury, and methyl jasmonate both in young and mature leaves (Thipyapong et al. 1997). Methyl jasmonate has been shown to increase PPO activity in some plant species like tobacco, poplar, and potato (Constabel and Ryan 1998), while several others do not respond to methyl jasmonate (Constabel and Ryan 1998). Further, additional mechanisms may be involved in elevation of PPO activities under stress conditions like (1) activation of pre-existing PPO forms or alteration in response to abiotic or biotic stress (Boss et al. 1995; Thipyapong et al. 1995; Soler-Rivas et al. 1997), (2) fatty acids released under stress from membrane lipids (Goldbeck and Cammarata 1981), (3) non-proteolytic mechanism (e.g., tolassin, a toxin from *Pseudomonas tolaasii*; Soler-Rivas et al. 1997), and (4) stimulation of PPO activity by phytohormones (Saluja and Sachar 1982).

Multiplicity of plant PPOs makes them a very special class of enzyme system with a very diverse range of characters dependent upon different conditions including temperature and pH. It is a challenging task to identify and characterize all the PPOs in a particular plant species as these mostly occur as multigene families having different substrate specificities, inhibitors, and activation processes. Further, the multiple PPOs also exhibit differential expression (both spatial and temporal) coupled with differential regulation both at transcriptional and translational levels.

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Reaction Features of Polyphenol Oxidases

4

Polyphenol oxidases (PPOs) are dinuclear copper-containing metalloproteins that are present and characterized in several higher plants (Flurkey 1989; van Gelder et al. 1997; Demeke and Morris 2002). PPOs are also known as catechol oxidase, catecholase, diphenol oxidase, *o*-diphenolase, phenolase, and tyrosinase (Martinez and Whitaker 1995). PPO is a broad term describing an enzyme capable of catalyzing several different phenols to produce *o*-quinones (Mathew and Parpia 1971; Whitaker 1994). Catechol oxidases (E.C.1.10.3.1) catalyze two reactions: the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones (Fig. 4.1). Oxygen is the primary oxidant in PPO-catalyzed reactions (Mason 1957; Mayer and Harel 1979; Vamos-Vigyazo 1981; Zawistowski et al. 1991). These two oxygen-consuming reactions are referred to as cresolase/monophenolase (reaction 1) and catecholase/diphenolase activity (reaction 2). Monophenolase or cresolase activity is not always present, and when both cresolase and catecholase activities are present, their ratio varies from 1 to 10 or may be even up to 40 (Brooks and Dawson 1966; Vamos-Vigyazo 1981).

Laccases (E.C.1.10.3.2) catalyze both *o*-diphenols and *p*-diphenols, producing their corresponding *o*- and *p*-quinones (Fig. 4.2; Nicolas et al. 1994). The ability of laccases to catalyze *p*-diphenols distinguishes it from catechol oxidases. The nomenclature of these phenol catalyzing enzymes is confusing since, apart from E.C.1.10.3.1 and E.C.1.10.3.2, a third one E.C.1.14.18.1 exists. This enzyme (E.C.1.14.18.1) is called as monophenol monooxygenase (tyrosinase) and corresponds to E.C.1.10.3.1; it always catalyzes the hydroxylation of monophenols (Nicolas et al. 1994). Though there is some confusion regarding the nomenclature, for the purpose of this book, we will use the broad term polyphenol oxidase (PPO).

PPOs are an important class of type 3 copper enzymes (Kaintz et al. 2015). These type 3 copper enzymes have two diamagnetic spin-coupled copper atoms (Lerch et al. 1986) in the highly conserved copper-binding domains: CuA and CuB. Each copper ion coordinates with three histidine residues, comprising the active site

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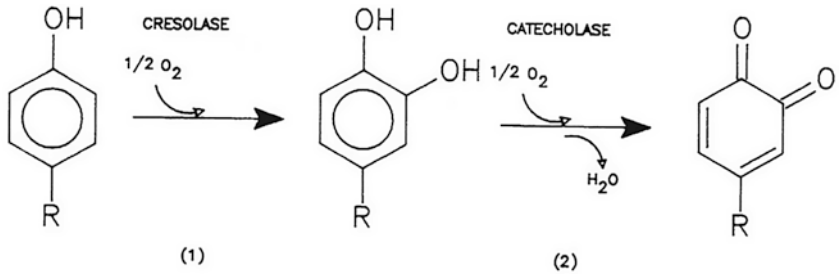
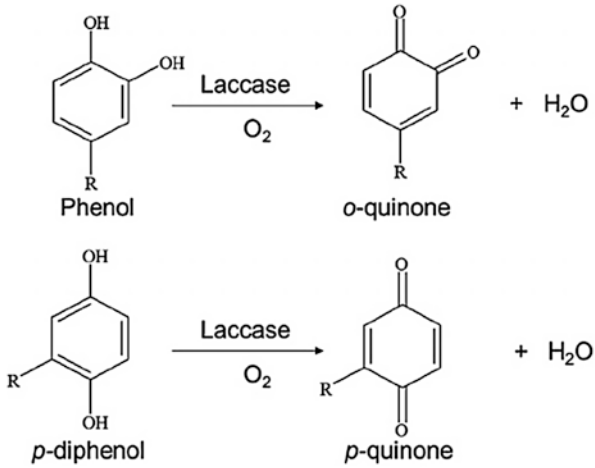


Fig. 4.1 Cresolase and catecholase activity of polyphenol oxidases (Source: Nicolas et al. 1994, with permission)

Fig. 4.2 Production of corresponding quinones by laccases using different phenols (Source: Nicolas et al. 1994, with permission)



(Klabunde et al. 1998). Between the two copper atoms, oxygen is bound in a "side-on" bridge mode (μ-η^2:η^2; Kitajima et al. 1989). Oxygen as the primary oxidant and copper as a prosthetic group are essential for PPO activity (Yoruk and Marshall 2003). The typical scheme of PPO reactions and nonenzymatic oxidative condensation of *o*-quinones is shown in Fig. 4.3. The phenol/oxygen is the substrate for PPO reaction, and BH_2, a diphenolic compound, acts as an electron donor (Fig. 4.3).

The lack of availability of BH_2 results in a delay in attaining a steady-state rate specifically for the monophenolase reaction (Kahn and Pomerant 1980; Sanchez-Ferrer et al. 1993). But if the PPO can produce BH_2, then a normal reaction rate is observed (Rodriguez-Lopez et al. 1992; Sanchez-Ferrer et al. 1993; Whitaker 1994; Lerch 1995). The rate of reaction is influenced by several factors including substrate, enzyme levels, temperature, and pH (Espin et al. 1997; Perez-Gilabert and Carmona 2000; Laveda et al. 2001; Yoruk and Marshall 2003).

In case of latent activity, detergents like sodium dodecyl sulfate (SDS) can be added to reduce the reaction delay (Jimenez and Garcia-Carmona 1996; Sojo et al.

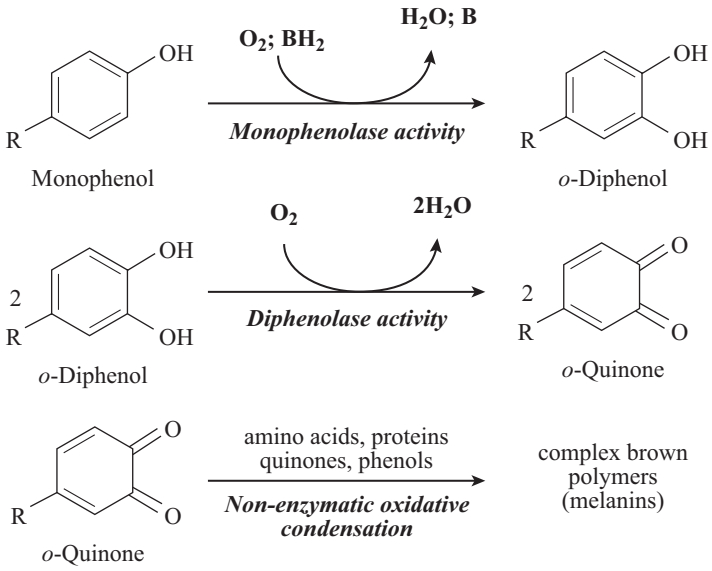


Fig. 4.3 Enzymatic and nonenzymatic condensation process of browning. BH_2 – *o*-diphenolic compound (Source: Yoruk and Marshall 2003, with permission)

1998). The hydroxylation reaction involves the transfer of one oxygen atom to monophenol and reduction of another to water (Mason 1957). The origin of oxygen in the monophenol was shown to be molecular oxygen but not water (Mason 1956).

The BH_2 is not required in the diphenolase activity (Fig. 4.3) as the already present *o*-diphenols are h to complete the reaction (Whitaker 1995) and produce the corresponding *o*-quinones. In the *o*-quinone-producing reaction, the two atoms of oxygen are reduced to water; due to this the diphenolase activity is also called as a "four-electron transfer oxidase" (Mason 1957). Enzyme concentration-independent, but pH and substrate concentration-dependent, lag phase has been reported for grape catecholase activity demonstrating the hysteretic nature of PPOs (Valero and Garcia-Carmona 1992, 1998).

The *o*-quinones generated as products of PPO reactions are themselves colored. But the typical brown discoloration of fresh or processed products is produced by *o*-quinones upon subsequent self-polymerization or their reaction with different functional groups (Feillet et al. 2000; Anderson and Morris 2003). The color varies in hue and intensity depending upon the originating phenols (catechin, yellow; DOPA, pink; chlorogenic acid, orange yellow), pH, and environmental factors (Pierpoint 1969a, b; Singleton 1987; Taylor and Clydesdale 1987; Rouet-Mayer et al. 1990). Further, the *o*-quinones generated are reactive compounds as they can react with another phenol producing dimers of the original phenolic compounds (Fig. 4.4; McDonald and Hamilton 1973; Singleton 1987; Young et al. 1987). Reoxidation of these dimers either enzymatically or by another *o*-quinone results in

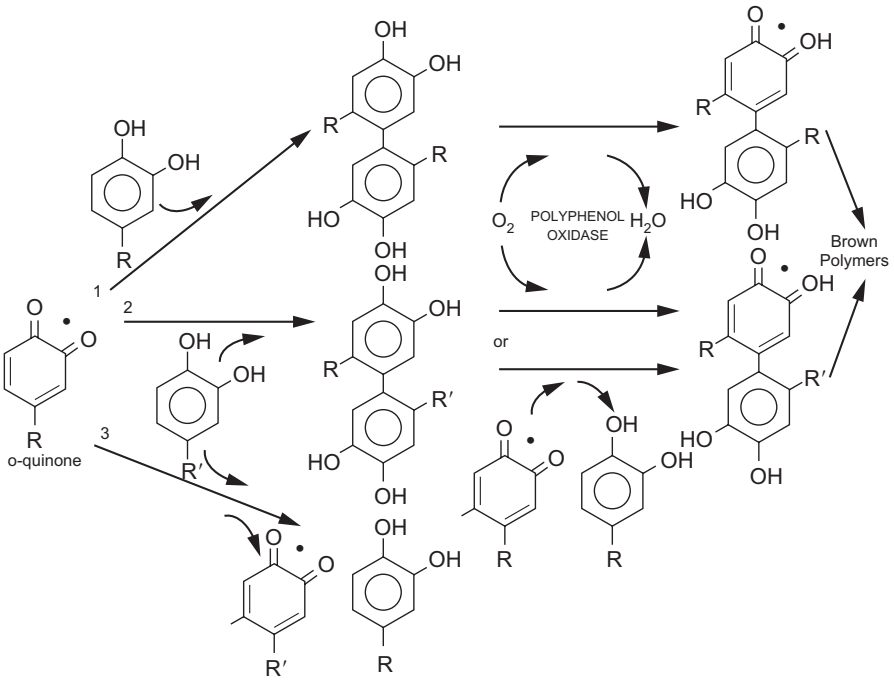


Fig. 4.4 The reactions of o-quinones with phenolic compounds (Source: Nicolas et al. 1994, with permission)

the formation of oligomers with totally different color properties (Cheynier and Moutounet 1992; Singleton 1987). Interestingly, the *o*-quinones can also react with a different phenol leading to a copolymer or produce the original phenol, generating another *o*-quinone through a coupled reaction (Fig. 4.4; Cheynier et al. 1989; Cheynier and Ricardo da Silva 1991; Oszmianski and Lee 1990).

Additionally, the *o*-quinones can also react with nonphenolic compounds (Fig. 4.5). Dehydroascorbic acid and phenol are the products of coupled oxidation reaction of *o*-quinones with ascorbic acid (reaction 1). Similarly colorless compounds along with phenol are formed with sulfites (reaction 2; Embs and Markakis 1965; Ponting 1960; Sayavedra-Soto and Montgomery 1986; Singleton 1987; Wedzicha 1984). Nucleophilic addition of *o*-quinones with thiol groups generates additional compounds (reactions 3 and 4). Cysteine either in bound (Cheynier et al. 1989; Henze 1956; Pierpoint 1969a, b) or free form (Dudley and Hotchkiss 1989; Mason and Peterson 1965; Friedman and Molnar-Perl 1990; Pierpoint 1966; Roberts 1959; Sanada et al. 1972) produces colorless compounds with *o*-diphenolic structure. Despite being *o*-diphenolic in structure, these compounds are not PPO substrates (Cheynier and VanHulst 1988; Richard et al. 1991; Sanada et al. 1972; Singleton et al. 1985) but can react with *o*-quinones forming dark-colored products. Further, addition or substitutions occur with amine (primary or secondary amine;

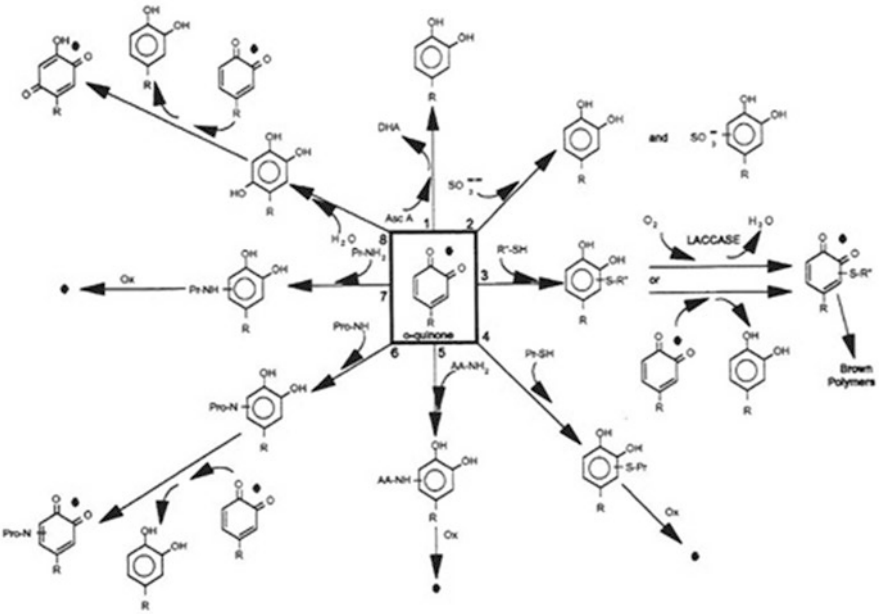


Fig. 4.5 The reactions of *o*-quinones with nonphenolic compounds. *Asc A* ascorbic acid, *DHA* dehydroascorbic acid, *AA-NH*$_2$ amino acids, *Pro-NH* proline, *Pr-SH* and *Pr-NH*$_2$ proteins, *RSH* thiol compounds (e.g., glutathione or cysteine), *Ox* further oxidation reactions by oxygen or *o*-quinones (Source: Nicolas et al. 1994, with permission)

reactions 5, 6, 7) and thiol (reaction 4) groups of proteins forming intra- or intermolecular cross-links (Nicolas et al. 1994; Matheis and Whitaker 1984; McMannus et al. 1985; Pierpoint 1985).

Triphenols formed by slow addition of water to *o*-quinones can be oxidized by PPOs or by excess quinones to *p*-quinones (reaction 8; Dawson and Tarpley 1963; Richard-Forget et al. 1992) mostly under acidic pH conditions (Garcia-Canovas et al. 1982; Garcia-Moreno et al. 1991; Jimenez et al. 1986; Richard-Forget et al. 1992). The stability or reactivity of *o*-quinones in these different reactions is highly variable and largely depends upon conditions (pH and temperature) and nature of phenols.

4.1 Mechanism of Enzymatic Browning

To control the process of enzymatic browning, we require chemical knowledge about PPO/substrate levels at different developmental stages of plant, reducing compounds, and polymerization/degradation reactions of *o*-quinones (Whitaker and Lee 1995). Further, it is essential to understand the difference between enzymatic and nonenzymatic (Maillard reaction) browning. Nonenzymatic reaction of

o-benzoquinones with O_2 generates complex compounds like indole-5,6-quinones which on further polymerization yield melanin. The *o*-benzoquinones can also react with other phenols (by Michael addition), amines, and thiols to form a variety of products including protein polymers (Wong et al. 1971; Matheis and Whitaker 1984).

4.1.1 PPO Reaction Mechanism in *N. crassa*

The mechanism of PPO action has been studied comprehensively in *N. crassa*, and this provided a plausible explanation to PPO's catalytic reaction (Fig. 4.6; Lerch 1983; Solomon et al. 1992).

The catalytic action of PPO was proposed based on the structure (both electronic and geometric) of the copper active site (Himmelwright et al. 1980; Lerch 1983; Wilcox et al. 1985; Solomon et al. 1992). This active site structure resembles that found in hemocyanin (Himmelwright et al. 1980; Gaykema et al. 1984). The active site is present in three different forms: *met*-PPO [Cu^{+2}], *deoxy*-PPO [Cu^{+1}], and

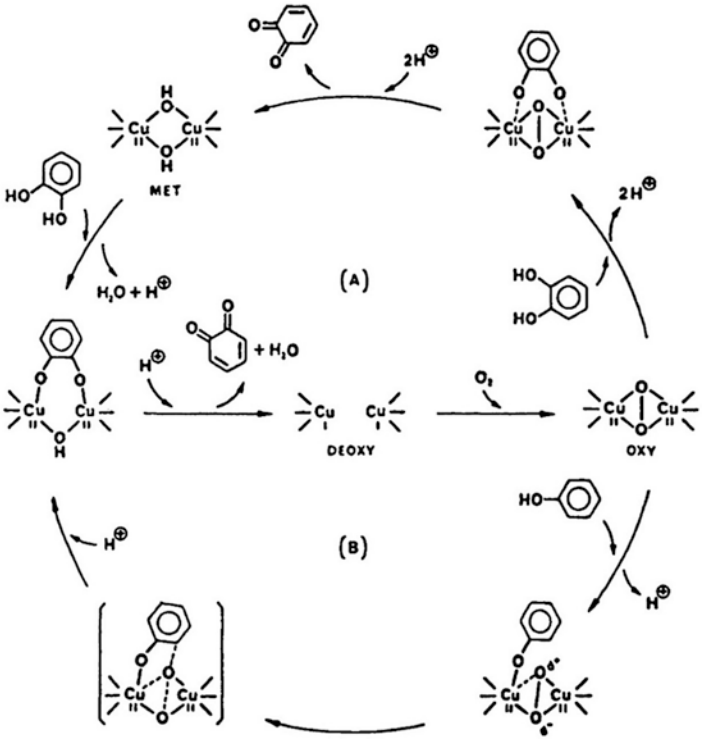


Fig. 4.6 The mechanism of PPO reaction for *N. crassa* PPO (Source: Whitaker and Lee 1995, Recent advances in chemistry of enzymatic browning, In: Enzymatic Browning and Its Prevention, Copyright 1995 American Chemical Society, with permission)

oxy-PPO [Cu^{+2}]. The *met*-PPO form is thought to be the resting form (Himmelwright et al. 1980; Solomon et al. 1992). The proposed mechanism of PPO action probably occurs by two separate pathways linked by a *deoxy*-PPO intermediate (Fig. 4.6). Figure 4.6a shows the dehydrogenation mechanism. First, the O_2 binds to the Cu (I) of *deoxy*-PPO to produce *oxy*-PPO. The bond distance between O_2 and Cu (II) groups is typical of a peroxide (Solomon et al. 1992). The copper (II) groups of *oxy*-PPO bind to the oxygen atoms of the *o*-diphenol to form the O_2/*o*-diphenol/PPO complex. The *o*-diphenol is oxidized to *o*-benzoquinone, and the PPO is reduced to its *met* state. Further, another *o*-diphenol molecule is attached to the *met*-PPO forming *o*-benzoquinone, and the enzyme itself gets reduced to *deoxy* state, completing the cycle.

The *o*-hydroxylation reaction of monophenols by PPO is shown in Fig. 4.6b. Monophenolic compounds can react with only *oxy*-PPO forming a ternary complex (Lerch 1995). Since in in vitro the reaction starts with the resting *met*-PPO, it must be reduced by a reducing compound to avoid lag period and to produce *deoxy*-PPO. The *deoxy*-PPO binds with O_2 to form *oxy*-PPO. The monophenolic compounds bind through an oxygen atom of their hydroxyl group with one of the Cu (II) groups of *oxy*-PPO resulting in O_2–monophenol–PPO complex. Further, an oxygen atom of the PPO complex is utilized in hydroxylation of *o*-position of the monophenol to give catechol. Subsequently, the catechol dissociates, and *deoxy*-PPO is generated completing the cycle. It has been shown that first oxygen followed by monophenol binds to the enzyme in an orderly sequential mechanism (Wilcox et al. 1985). On contrary, the binding of *o*-diphenol and oxygen to *deoxy*-PPO was indicated to be a random sequential mechanism (Wilcox et al. 1985), but if the recycling steps of proximal pathway are considered, the mechanism appears ordered (Sanchez-Ferrer et al. 1995). An ordered sequential Bi Bi mechanism is generally assumed during diphenolase activity with oxygen being the first substrate (Janovitz-Klapp et al. 1990; Whitaker 1994). Further, NMR studies in apple, pear (Espin et al. 1998), and mushroom (Espin et al. 2000) have demonstrated that monophenols with a high electron-donor side chain are oxidized more rapidly. But the size of the side chain substituent had insignificant effect on the enzymatic velocity owing to the low nucleophilic power of the monophenols. Interestingly, the electron donor capabilities of o-diphenols have negligible impact on the reaction rate, whereas the size of side chain (steric effect) had significant effect (Espin et al. 1998, 2000). Different models explaining monophenolase and diphenolase activities of plant PPOs have been proposed based on the kinetic aspects, different enzymatic states, and the reaction pathway of *N. crassa* (Rodriguez-Lopez et al. 1992, 1993).

4.1.2 Reaction Mechanism of Catechol Oxidase from *Ipomoea batatas*

The crystal structure of catechol oxidase from *Ipomoea batatas* (*ib*CO) was successfully determined by Klabunde et al. (1998). Klabunde et al. (1998) have given a pathway that explains the catechol oxidation by catechol oxidase through the

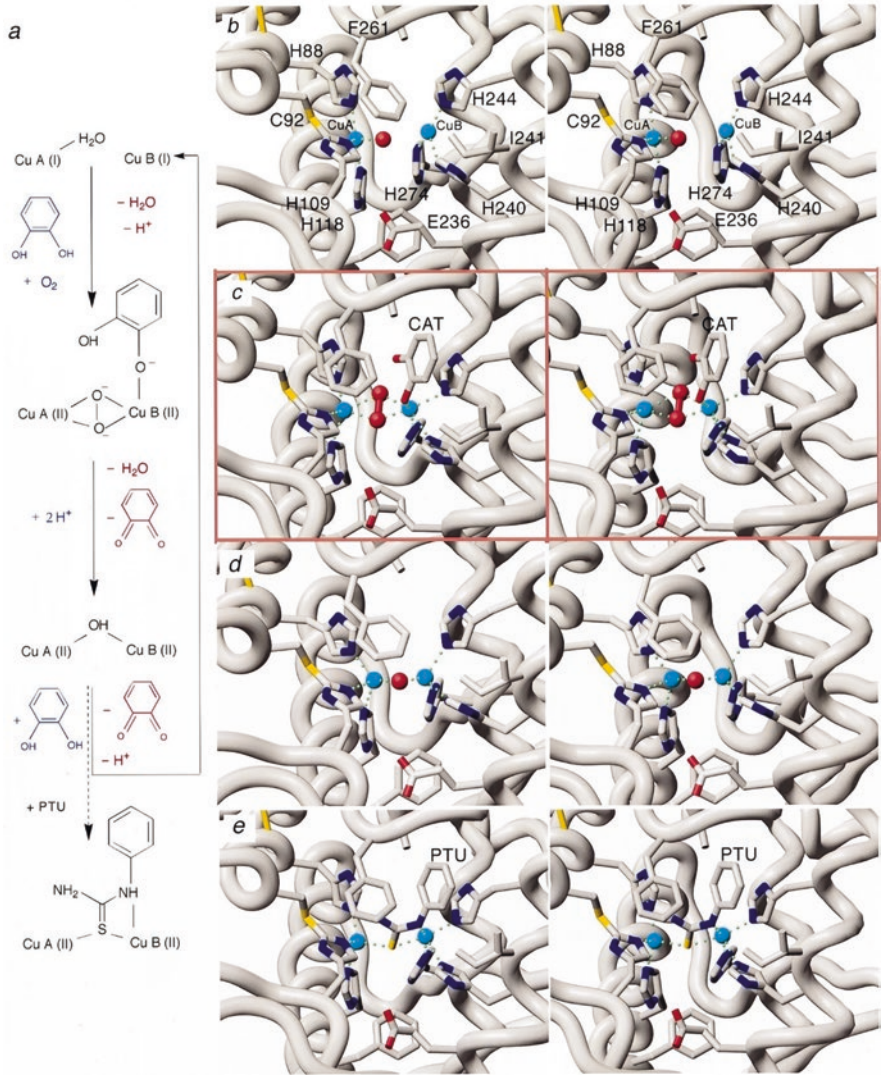


Fig. 4.7 Proposed reaction pathway of catechol oxidase (**a**) The reaction pathway of catechol oxidation by catechol oxidase is shown in three steps: two molecules of catechol (or derivatives thereof) are oxidized, coupled with the reduction of molecular oxygen to water. The binding mode of PTU is displayed at the bottom. (**b**–**d**) The three-dimensional structures for each of the three reaction steps, as derived from crystallographic analysis. Note that the ternary catechol oxidase–O_2^{2-}–CAT complex shown in (**c**) is a model, guided by (**e**), the binding mode observed for the inhibitor PTU 9 (Source: Klabunde et al. 1998, Nat Struct Mol Biol, 5: 1084–1090)

four-electron reduction of the molecular oxygen to water (Fig. 4.7a). In the suggested pathway, the solvent molecule bonded to the CuA site in the reduced form is replaced by dioxygen, and it binds to the dicuprous metal center (Eicken et al. 1998;

Wilcox et al. 1985; Solomon et al. 1996; Fig. 4.7b, top of 4.7a). It seems that oxygen binding to the reduced enzyme precedes the binding of catechol since incubation of high molar excess of catechol with dithiothreitol reduced crystals showed no catechol binding indicating low binding affinity of catechol to the reduced dicuprous (Cu[I]-Cu[I]) form. The spectroscopy results indicate the binding of molecular oxygen as peroxide in a bridging side-on μ-η^2:η^2 mode with a Cu–Cu separation of 3.8 Å (Eicken et al. 1998). A similar binding mode was observed with a Cu–Cu separation of 3.6 Å for oxygenated hemocyanin from *Limulus polyphemus* (Magnus et al. 1994).

The catechol binding to the dicopper center is permitted due to the rotation of the side chain of Phe261 residue. Based on the phenylthiourea (PTU)–catechol oxidase inhibitor complex (Fig. 4.7e; bottom of Fig. 4.7a), it was suggested that simultaneous binding of catechol and oxygen is feasible. Further, based on the same inhibitor complex, Klabunde et al. (1998) proposed a mono-dentate binding model (Fig. 4.7c; second from top in Fig. 4.7a). According to this model, the catechol binds after the deprotonation (assisted by Glu236) of one of the two hydroxyl groups to CuB. As per this model, CuB is six coordinated with a tetragonal planar coordination by His240, His244, and two oxygen molecules. The catechol and His274 occupy the axial positions in this distorted octahedral coordination favored by Cu(II) with nine d-electrons. But CuA is in tetragonal–pyramidal coordination with His88, His118, and O_2^{2-} in equatorial. One axial position is occupied by His109, and other non-solvent accessible sites are vacant.

In the suggested catechol oxidase–O_2^{2-}–catechol complex, two electrons are transferred from the substrate to the peroxide leading to the protonation of the peroxide and cleavage of the O–O bond. The promotion of loss of water and departure of *o*-quinone could be due to donation of a proton by Glu236 and the second non-coordinating hydroxyl group of the substrate. The protonation brings the catalytic site into the resting hydroxide-bridged dicupric state (Fig. 4.7d; second from the bottom in Fig. 4.7a). A second catechol molecule can revert the active site to dicuprous form by reducing the hydroxide-bridged dicupric state. This step is supported by results from o-diphenolase activity of tyrosinase (Wilcox et al. 1985; Solomon et al. 1996) and as well as data on catechol oxidase of sweet potato (Klabunde et al. 1998). Further, mono-dentate binding of a diphenolic substrate to CuB could probably reduce the dicupric (Cu[II]-Cu[II]) state back to dicuprous (Cu[I]-Cu[I]) form, thereby repeating the catalytic cycle.

4.1.3 Reaction Mechanism of Aurone Synthase from *Coreopsis grandiflora*

Based on the binding of a substrate to CuA site in bacterial tyrosinase (*Bacillus megaterium*; *bm*Tyr), it was proposed that the monophenolic substrates perform a rotation during hydroxylation reaction (Goldfeder et al. 2014). Further, the authors speculated that the same phenomenon of substrate binding to CuA will also occur in plant catechol oxidases. Therefore, based on these speculations, it was concluded

that hydroxylation reaction is not possible in plant catechol oxidases mostly due to the bulky gate residue in addition to the rigidity of CuA site (thioether bond) hindering the substrate rotation. Interestingly, this does not explain the hydroxylation activity of *Juglans regia* tyrosinase (*jr*Tyr; Zekiri et al. 2014a; Escobar et al. 2008) and AUS1 despite both of them possessing bulky gate residue and thioether bond. Furthermore, the lack of monophenolase activity of *Aspergillus oryzae* catechol oxidase (*ao*Co) toward tyrosine was never explained (Hakulinen et al. 2013).

The classical molecular dynamics (MD) simulations could not provide information regarding the geometry of the substrate–copper binding scenario. But the hydrophobic interactions of the substrate with Phe273, the gate residue, and His256 direct the B-ring to the catalytic site wherein the substrate's *o*-diphenolic oxygen atoms are almost symmetrically located between the dicopper ions (Fig. 4.8a, b).

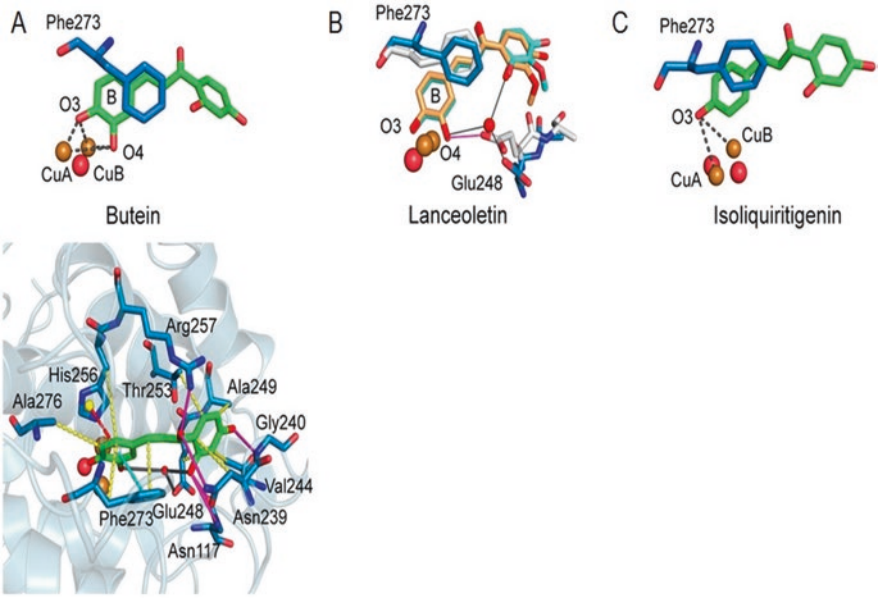


Fig. 4.8 (**a**) MD simulation snapshot of AUS1 in complex with butein. The reactive oxygen atoms at the B-ring of butein (O3, O4) are almost symmetrically located between CuA and CuB. The enzyme–substrate interactions are visualized by magenta lines [hydrogen bonding], *yellow dashes* [hydrophobic interactions], *cyan dashed lines* [π–π stacking], *red dashes* [cation–π interactions], and *gray lines* [water bridges]. (**b**) Superimposition of snapshots of AUS1 with lanceoletin. Residue Glu248 coordinates an oxygen atom of the A-ring through the formation of a water bridge [*blue sticks*; water bridges, *gray lines*] or one reactive oxygen atom [O4, B-ring] directly (*white sticks*; hydrogen bonding, *magenta lines*]. (**c**) MD simulation snapshot of AUS1 in complex with isoliquiritigenin (Source: Molitor et al. 2016, PNAS 113(13): E1806–15)

The position of oxygen atoms in a way resembles to the butterfly distorted oxygen atoms of *oxy*-form probably indicating the possibility of this coordination.

Further, stabilization of isoliquiritigenin in a particular position allows the hydroxylation in the *ortho* position to the copper-coordinating hydroxyl group (Fig. 4.8c). Based on these observations coupled with the discovery of hydroxylation activity of AUS1 and *jr*TYR (Bijelic et al. 2015; Zekiri et al. 2014a, b), Klabunde et al. (1998) proposed a novel mechanism for catalysis of phenolic substrates by plant PPOs (Fig. 4.9). As per the proposed novel mechanism, no substrate rotation is required since the substrate is symmetrically coordinated to the two copper atoms, thereby removing the contradictions of the earlier proposed mechanisms.

Further, the simulation studies of tyramine in the catalytic site of AUS1, *jr*TYR, and L244R mutant of *jr*TYR intensely indicate that the residue at position of Arg257 (Fig. 4.8) is a crucial residue for accepting tyramine as a plant PPO substrate. However, the interactions of fungal and bacterial PPOs will differ significantly due to lack of bulky gate residue and direct binding of substrates to CuA (Goldfeder et al. 2014). Interestingly, the MD simulations with tyramine has revealed that it is destabilized in the catalytic center of plant PPOs by an arginine residue at this key position (AUS1, L244R mutant of *jr*TYR), but a leucine at this position stabilizes it (*jr*TYR). Further, *Vitis vinifera* catechol oxidase (*vv*CO) has a leucine in this critical position and was shown to possess weak tyrosinase activity toward tyramine (Fronk et al. 2015). Despite showing weak tyrosinase activity, kinetic data of *vv*CO and *jr*TYR clearly indicates that tyramine may not be the natural substrate of *vv*CO (Km of vvCO = 7.7 mM, Fronk et al. 2015; Km of jrTYR = 0.274 mM, Bijelic et al. 2015). Klabunde et al. (1998) have demonstrated that monophenolase activity of plant PPOs is mainly determined by the ability of the enzyme to stabilize the monophenol in its active site or not.

Despite the advances made in understanding the reaction mechanism(s) of plant PPOs, interdisciplinary research involving metabolomic, transcriptomic, and proteomic analyses is required to clarify the role of PPOs. The differences in the substrate binding residues around both the copper sites differ significantly within the PPO families (Tran et al. 2012; Dirks-Hofmeister et al. 2014) probably indicating the presence of unique natural substrates. Therefore, the challenges would be to classify the PPOs based on their substrate binding residues, find their natural substrates, and confirm the involvement of plant PPOs in secondary metabolism as already in some cases.

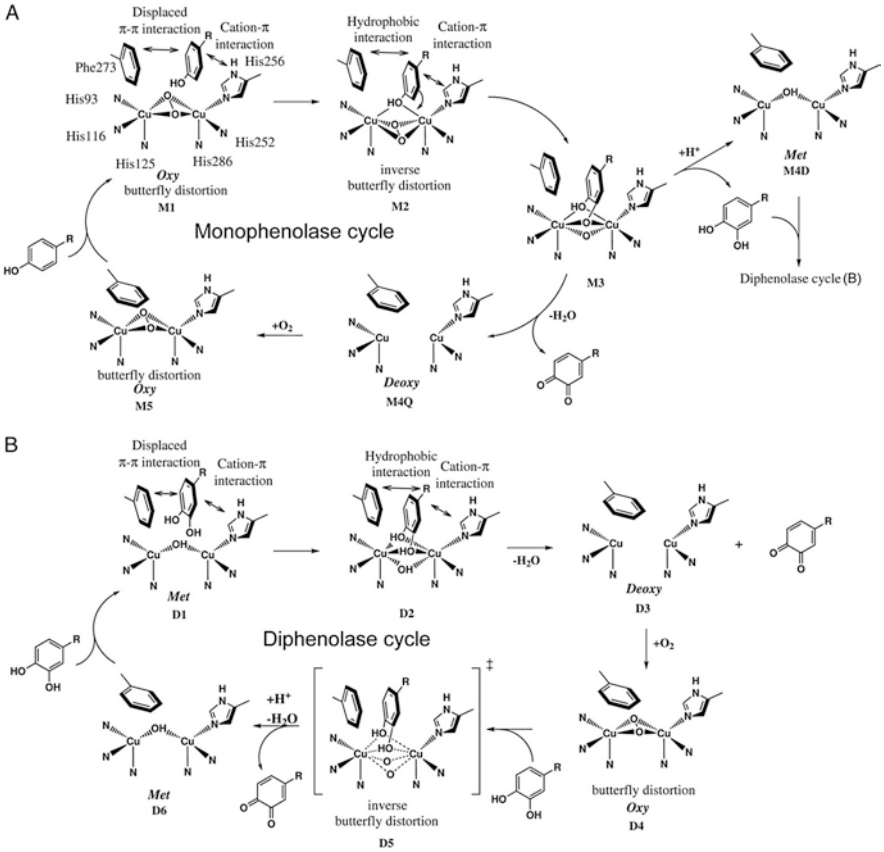


Fig. 4.9 Proposed catalytic reaction mechanism of plant PPOs. (**a**) Monophenolase cycle: The monophenolic substrate is guided to the binuclear copper site [oxy-form, $Cu(II)_2O_2$, μ-η2:η2 peroxide complex] by displaced π–π interactions with the tilt out gate residue Phe273 and cation–π interaction with the CuB binding histidine His256 (M1). When the substrate binds to the copper atoms, hydrophobic interactions with the substrate's para-substituent become important additionally. The reactive substrate oxygen atom binds equally to both copper ions (M2), and the peroxide ligand is transferred to an inverse butterfly distortion. Nucleophilic attack of the Cu_2O_2 moiety by the substrate results either in an o-diphenolic product and the *met*-form [M4D, $Cu(II)_2OH$] or in an o-quinone and the deoxy-form [M4Q, $Cu(I)_2$]. During product release, the gate residue swings back to its preferred position. Finally, oxygen uptake closes the catalytic cycle (M5). (**b**) Diphenolase cycle: The principles of the diphenolase cycle are similar to the monophenolase cycle described in A. The reactive substrate oxygen atoms bind equally to both copper ions of the met-form (D1, D2), and the o-quinone is released after electron transfer to the binuclear copper site resulting in the deoxy-form (D3). Oxygen uptake results in the *oxy*-form (D4), and substrate binding results in an inverse butterfly distortion in the transition state (D5). The catalytic cycle is closed by the release of an o-quinone resulting in the met-form of the binuclear copper site (Source: Molitor et al. 2016, PNAS 113(13): E1806–15)

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Function(s)/Role(s) of Polyphenol Oxidases

5

Plants under native conditions are exposed to different abiotic and biotic stresses that impact the plant fitness either individually or together. Under majority of situations, the plants possess a diverse range of defense traits that help in reducing or eliminating the effects of these stresses. The secondary metabolites that include defense-related proteins play a major role in plant defense. Generally, spatial and temporal differences are observed with occurrence of biotic and abiotic stresses. Additionally, multiple stresses might impact the plant growth simultaneously; therefore, plant defense mechanisms must exhibit plasticity to combat these different stresses. The flexibility of secondary metabolites is due to (i) their role in defense against several stresses both biotic and abiotic and (ii) they are inducible, i.e., secondary metabolites are only expressed or expressed under stress (Thipyapong et al. 2007).

The physiological and biochemical roles of plant PPOs have always been of interest to researchers of different disciplines. Polyphenol oxidases (PPOs) catalyze the oxidation of monophenols/*o*-diphenols to *o*-quinones, and the secondary reactions of o-quinones produce undesirable browning that occurs as a result from senescence, wounding, and pathogen infection (Mayer and Harel 1991; Friedman 1997). PPOs have been implicated for their potential role in (i) phenylpropanoid pathway (Kojima and Takeuchi 1989); (ii) Mehler reaction, electron cycling, and oxygen regulation (Vaughn et al. 1988; Trebst and Depka 1995); (iii) flower petal coloration (Nakayama et al. 2000); (iv) plant defense (Li and Steffens 2002; Thipyapong et al. 2004; Wang and Constabel 2004); and (v) other biosynthetic processes (Mayer 2006). Despite their probable involvement in different processes, there is a lack of clarity or specificity of PPOs with regard to their role/function (Mayer and Harel 1979). But during the past decade, there has been significant progress in deciphering the role of PPOs especially in plants which will be discussed in detail.

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5.1 Role of PPOs in Plant Defense

5.1.1 Role of PPOs in Plant Resistance Against Pathogens

A potential role in plant defense has always been the major focus of PPO research. PPOs have been implicated in plant defense mostly due to the appearance of their reaction products upon pathogen or insect attack, wounding, and inducibility in response to different stresses (Mayer and Harel 1979; Constabel et al. 1995; Thipyapong and Steffens 1997; Maki and Morohashi 2006). The hypothesis of PPOs' role in plant defense has mostly been supported by correlative studies. Pathogen-induced PPO activity coupled with high PPO levels often associated with increased pathogen resistance among cultivars underscores the role of PPOs in plant defense against pathogens (Chen et al. 2000; Deborah et al. 2001; Raj et al. 2006). Raj et al. (2006) through inoculation and native PAGE studies, tissue printing analysis, and transcript accumulation studies were able to positively demonstrate the involvement of PPOs in pearl millet [*Pennisetum glaucum* (L.) R.Br] defense against down mildew caused by *Sclerospora graminicola* (Sacc.) Schroet. Potato varieties with enhanced PPO levels showed better tolerance to soft rot disease caused by *Pectobacterium atrosepticum*, *Pectobacterium carotovorum* subsp. *brasiliensis*, and *Dickeya* spp. (Ngazee et al. 2012). Similarly, PPO could be used as a marker against *Ralstonia solanacearum* and *Xanthomonas axonopodis* pv. *vesicatoria* causing bacterial wilt and leaf spot disease, respectively (Kavitha and Umesha 2008; Vanitha et al. 2009). PPO activity was induced in the whole seeds and also in dissected parts including lemma, palea, and caryopses of wild oats when challenged with *Fusarium avenaceum* strain *F.a.*1 (Fuerst et al. 2014). The PPO induction in nonliving hull (lemma and palea) was surprising, but activation of PPOs in hulls may be possible similar to as in caryopses. The authors hypothesize, and to some extent their results support the simultaneous activation of mature, latent PPO, followed by their release, probably by proteolytic cleavage, as a part of defense mechanism against pathogen attack. Fuerst et al. (2014) suggested that multiple defense enzymes are induced by *F.a.*1 in wild oat caryopses. However, significant progress in understanding the molecular biology of PPOs has resulted in a more direct evidence of their role in plant defense. Expression of specific PPO genes and manipulation of the PPO expression levels under different stress conditions have enabled us to understand the PPO's role in plant defense and to an extent the mechanism(s) involved.

Transgenic approach was utilized in tomato to study the role of PPOs in plant disease. Transgenic tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) plants were produced by overexpressing a potato (*Solanum tuberosum* L.) leaf PPO cDNA using *Agrobacterium tumefaciens*-mediated transformation (Li and Steffens 2002). Attempts to overexpress tomato PPO cDNA PPO-B in tomato were unsuccessful; therefore, a potato leaf PPO was utilized in transforming tomato. The PPO transcript and activity levels in the transgenic tomato plants were ~30-fold and five- to tenfold higher, respectively, compared to the control plants. The endogenous phenolic substrate pool was oxidized at a faster rate in transgenic tomato plants compared to the control plants. This was evident, as 78% and 16% of chlorogenic acid (most

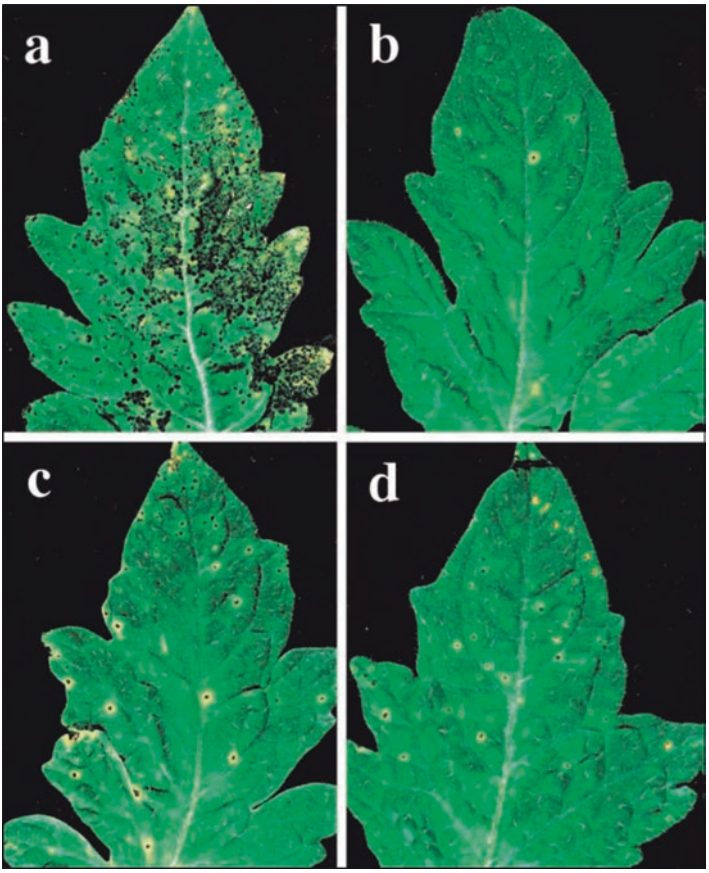

Fig. 5.1 (a–d) Tomato transgenic plants exhibiting resistance to *P. syringae* of node 4 leaves. (**a**) Non-transformed plant (**b**–**d**) transgenic lines S-28, S-18, and S-84 (Source: Li and Steffens 2002, with permission)

abundant *o*-dihydroxyphenol in tomato leaves) was not recoverable 30 min after homogenization in transgenic and control plants, respectively. Further, as expected, the transgenic tomato plants exhibited greater level of resistance to *P. syringae* compared to the non-transformed control plants (Fig. 5.1). The transgenic lines showed greater suppression of disease symptoms, with ~15-fold fewer lesions per leaf area compared to the control plants. Transgenic lines demonstrating lower lesions also exhibited significant reduction (>100-fold) of bacterial growth in the infected leaf tissues.

Li and Steffens (2002) utilized overexpression studies to demonstrate the PPOs' role in plant defense; interestingly, downregulation studies (antisense) by Thipyapong et al. (2004) yielded similar results. Thipyapong et al. (2004) introduced chimeric antisense potato PPO cDNA constructs into tomato (*Lycopersicon esculentum* L.) plants to study the resistance response of tomato plants to

Pseudomonas syringae. The antisense PPO downregulated all the members of PPO gene family in tomato (seven – A, A′, B, C, D, E, and F). The transgenic tomato plants with downregulated PPO genes exhibited reduced PPO activity (by a factor of ~40) accompanied by decreased browning capability and secondary reactions of quinones. Further, transgenic plants with reduced PPO expression levels demonstrated higher susceptibility to *P. syringae*. The level of susceptibility of mature tomato plants to the pathogen depended upon the developmental stage, i.e., young leaves are more susceptible. Interestingly, antisense PPO transgenic plants were phenotypically similar to non-transformed plants under controlled growth conditions. The germination rate, leaf traits, flowering time, pollen viability, seed set, and fruit production were comparable in both types of plants.

As plants develop new defense mechanisms, in parallel, the pathogens evolve new ways or strategies to overcome these plant defenses. Interestingly, some plants like dandelion (*Taraxacum officinale*) have developed very successful tactics, making them tolerant to most of the disease-causing pathogens. Dandelion is a weed widely distributed in America, Europe, and Asia. It is used both as a food (Escudero et al. 2003) and medicinal plant (Stewart-Wade et al. 2002). Dandelion exhibits high PPO activity; therefore, the potential role of PPOs in dandelion resistance against pathogens was analyzed using RNAi technology (Richter et al. 2012). The potential association of dandelion PPOs (five isozymes) in plant defense was studied against *Botrytis cinerea* and *Pseudomonas syringae* pv. tomato. Among the five PPOs, only *ppo-2* was induced, especially around the lesions produced by *B. cinerea*. The transgenic dandelion plants were produced by *Agrobacterium tumefaciens*-mediated leaf disk transformation technique. The RNAi silencing specifically of *ppo-2* reduced its expression level and simultaneously increased the susceptibility of dandelion plants to *P. syringae* pv. tomato. Postinoculation studies after 4 days exhibited bacterial populations to be 28- to 242-fold in *ppo-2* RNAi transgenic lines compared to the wild-type plants. Further, to study the antimicrobial potential of dandelion *ppo-2*, it was expressed in *Arabidopsis thaliana*, plant in which no genes coding for PPOs have been identified (Mayer 2006). Interestingly, leaf extracts from transgenic *A. thaliana* (expressing dandelion *ppo-2*) plants demonstrated substrate-dependent antimicrobial activity against *P. syringae* pv. tomato, whereas the leaf extracts of wild-type plants did not.

5.1.2 Role of PPOs in Plant Resistance Against Insect Pests

Though PPOs are produced extensively in several plant species, the ecological and physiological role of PPOs in plants is still not very well understood (Mayer 2006). But PPOs have been implicated in plant defense against insect pests (Felton et al. 1989; Gatehouse 2002). The involvement of PPO in defense against leaf-eating pests was suggested by Felton and his team; they demonstrated an inverse relation of *Heliothis zea* (corn earworm) and PPO levels in tomato (Felton et al. 1989). Studies by Felton et al. (1989, 1992) indicated the reduction in insect performance due to binding of *o*-quinones to amino acids in insect gut and subsequent lowering

Table 5.1 Herbivore-induced PPO upregulation in plants with unknown effects on insects

Family/plant	Insect species	Induction	Reference
Betulaceae			
Black alder (*Alnus glutinosa*)	*Agelastica alni*	~3X	Tscharntke et al. (2001)
Fabaceae			
Common bean (*Phaseolus vulgaris*)	*Melanoplus differentialis*	~2X	Alba-Meraz and Choe (2002)
Soybean (*Glycine max*)	*Spissistilus festinus*	~1.6X	Felton et al. (1994)
Salicaceae			
Hybrid poplar (*P. trichocarpa* x *P. deltoides*)	*Malacosoma disstria*	~12X	Constabel et al. (2000)
Solanaceae			
Tomato (*Solanum lycopersicum*)	*Leptinotarsa decemlineata*	~2X	Felton et al. (1992)
Potato (*Solanum tuberosum*)	*Leptinotarsa decemlineata*	~3–7X	Kruzmane et al. (2002)
Theaceae			
Tea (*Camellia sinensis*)	*Helopeltis theivora*	~2–3X	Chakraborty and Chakraborty (2005)

Adapted from Constabel and Barbehenn (2008)

of their nutritional value. An important discovery demonstrating the anti-herbivore role of PPO in tomato was reported by Constabel et al. (1995). They have shown that leaves of transgenic tomato plants overexpressing a prosystemin gene encoding a precursor of systemin (involved in tomato wound signaling) possessed enhanced PPO levels (up to 70-fold) compared to the wild-type plants. Additionally, the PPO levels in young tomato plants exposed to methyl jasmonate (MeJA) were equivalent to the levels observed in prosystemin overexpressing plants. PPOs are assumed to have a role in plant defense against pests since PPOs are induced by various signals (in tomato) along with other proteins like protease inhibitors (PI), arginase, and threonine deaminase that are anti-herbivore in nature (Bergey et al. 1996; Chen et al. 2005). Similarly, PPOs and PIs have been shown to be upregulated in tobacco (Constabel and Ryan 1998; Ren and Lu 2006) and poplar species (Constabel et al. 2000; Haruta et al. 2001) by MeJA and wound and herbivore stress. Genomic experiments in hybrid poplar have demonstrated upregulation of several additional potential defense-related genes involved in herbivore defense underscoring the complex nature of plant defense (Christopher et al. 2004; Ralph et al. 2006; Major and Constabel 2006). The induction of PPOs by herbivores has been shown in a wide array of plant taxa (Table 5.1), and this has been interpreted as a direct response of plant against insect attack. But the impact of the induced PPOs in these plant species on herbivores is not known. Interestingly, much stronger evidence indicating a potential impact of enhanced PPO on herbivores was studied mostly in Solanaceae species with limited range of insect pests (Table 5.2). Though a stronger correlation was established through these studies, a specific impact of induced PPO was still not possible. Transgenic approach is the best way to study the direct role of PPO in plant defense response against insect pests. The PPO levels can easily be

Table 5.2 PPO induction in plants with an impact on insect pests

Plant	Inducing agent	Induction	Effect	Herbivore	References
Solanaceae					
Tomato	*Helicoverpa zea*	~2X	(−)	*Spodoptera exigua*	Stout et al. (1998)
Tomato	*Helicoverpa zea*	~2X	(−)	Spider mite	Stout et al. (1998)
Tomato	Jasmonic acid	~3X	(−)	*Trichoplusia ni*	Thaler et al. (2002)
Tomato	Jasmonic acid	~3X	(−)	Thrips	Thaler et al. (1999, 2002)
Tomato	Jasmonic acid	~3X	(−)	Spider mite	Thaler et al. (2002)
Tomato	Jasmonic acid	~5X	(−)	*Spodoptera exigua*	Thaler et al. (1999)
Tomato	*Macrosiphum euphorbiae*	Decreased	(+)	*Spodoptera exigua*	Stout et al. (1998)
Tomato	Jasmonic acid	~2X	(−)	*Manduca sexta*	Redman et al. (2001)
Tobacco (wild)	Clipped sagebrush	~4X	(−)	Field damage by L and O[a]	Karban et al. (2000)
Potato (wild)	High constitutive PPO	~4–7X	(−)	*Leptinotarsa decemlineata*	Castanera et al. (1996)
Betulaceae					
Mountain birch	*Epirrita autumnata*	~3X	(−)	*Epirrita autumnata*	Ruuhola and Yang (2006)

Adapted from Constabel and Barbehenn (2008)
[a]Lepidoptera and Orthoptera

individually manipulated through transgenic approach, thereby enabling the researcher to understand the dynamics of plant defense against herbivory.

Interestingly, other correlation studies between PPO activity and resistance to insect pests using cultivars with varying levels of resistance or PPO-treated leaves gave mixed response. Potato genotype with high PPO activity demonstrated increased resistance to *Leptinotarsa decemlineata* (Colorado potato beetle; Castanera et al. 1996), while higher levels of PPO in coffee leaves had no impact on resistance to *Leucoptera coffeella* (coffee leaf miner; Melo et al. 2006). Similarly, an eightfold increase in PPO levels did not have any effect on *Lymantria dispar* caterpillars (Barbehenn et al. 2007). But a negative correlation between tomato leaf/fruit PPO levels and growth rate of *Helicoverpa zea* caterpillars was recorded (Felton et al. 1989). Higher PPO levels had a positive impact on the performance of *Manduca quinquemaculata* in tobacco (Kessler and Baldwin 2002). The ambiguous correlations of PPO levels with defense could be due to the multigene nature of PPO gene families. To better understand the role of PPO genes in plant defense, a comprehensive study of each member of a PPO gene family in a particular plant/crop needs to be carried out. Overall, diverse expression patterns of PPO observed in tomato (Hunt et al. 1993; Steffens et al. 1994) and eggplant (*Solanum melongena*;

Shetty et al. 2011) to different signals demonstrate that PPOs may be involved in other stress-related functions.

The PPO-overexpressing lines of tomato provided the strongest direct evidence for a defensive role of tomato PPOs against insect pests (Thipyapong et al. 2007; Bhonwong et al. 2009). The role of PPO in defense against herbivores was evaluated against *Helicoverpa armigera* (cotton bollworm), *Spodoptera litura* (common cutworm), and *Spodoptera exigua* (beet armyworm). Cotton bollworms exhibited increased foliage consumption and weight gains when fed on transgenic tomato lines with suppressed PPO genes (SP) compared to those fed on transgenic lines overexpressing PPO (OP) or non-transformed lines (NT). Further, a negative correlation was observed between PPO activity and weight gain/foliage consumption underscoring the role of PPO in insect resistance. Interestingly, beet armyworm also exhibited similar behavior when fed on OP or SP lines. Similarly, *Leptinotarsa decemlineata* [Say] (Colorado potato beetles) fed on antisense PPO transgenic tomatoes also demonstrated weight gain, foliar consumption, and survival (Thipyapong et al. 2007). Transgenic approach was also used in poplar to demonstrate the direct role of PPO in plant defense against herbivores. Transgenic *Populus* lines overexpressing leaf PPO gene were utilized to study the roles of PPO against *Malacosoma disstria* (forest tent caterpillars; Wang and Constabel 2004). The leaf disk bioassay studies showed reduced weight gain and higher mortality rates among caterpillars fed on PPO overexpressing transgenic lines compared to larvae feeding on control leaves. Interestingly, the caterpillar's growth was effected only when old egg masses were used. The transgenic studies in tomato and poplar provide strong evidence for a direct role of PPOs in resistance against herbivores.

5.1.3 PPO Regulation in Response to Stress

The defense mechanisms against pest or pathogen attack are usually a complex network (Macheix et al. 1990; Johal et al. 1995; Yoruk and Marshall 2003). The constitutive expression of PPO in plant development suggests that PPO could provide protection to developing parts against disease or pest injury (Yoruk and Marshall 2003). The abundant PPO in glandular trichomes of *Solanum* and *Lycopersicon* species is involved in PPO-mediated oxidative polymerization of trichome exudates resulting in entrapment and death of the insects (Kowalski et al. 1992). The PPOs could be induced either locally (Boss et al. 1995; Ray and Hammerschmidt 1998), systematically (Thipyapong et al. 1995), or both (Thipyapong and Steffens 1997) for plant protection. Further, wound-inducible PPOs have been reported from different plant species including tomato and potato (Yoruk and Marshall 2003). The octadecanoid-signaling pathway products like systemin and MeJA activate the synthesis of PPO in wounded tomato plants (Constabel et al. 1995). The in vivo activation or alteration of latent PPO forms in response to injury could also lead to enhanced PPO levels (Boss et al. 1995; Thipyapong et al. 1995). The PPOs could also be activated by free fatty acids released under stress (Goldbeck and Cammarata 1981). Non-proteolytic activation mechanism

(Soler-Rivas et al. 1997), appearance of additional multiple forms (Mari et al. 1998; Saluja and Sachar 1982), and phytohormone-mediated PPO activity (Saluja and Sachar 1982) may be responsible for higher PPO levels.

5.1.4 Mechanism of PPO Action in Plant Defense

Several studies discussed demonstrate the role of PPO in plant defense against pests and pathogens. Though PPOs have been implicated in plant defense, studies exhibiting their mode of action are limited. PPOs' role in plant protection is essentially based on the control of spread of infection by the reaction products of PPOs. The most possible mechanisms through which PPO might impact plant defense against pests and pathogens are:

1. The *o*-quinones: reaction products of PPOs could modify the proteins by reacting with different compounds including amino, phenolic, and sulfhydryl (Mason and Peterson 1965; Matheis and Whitaker 1984), thereby reducing the nutritional value of proteins to insect pests (Felton et al. 1989; Felton and Duffey 1992).
2. Redox cycling of *o*-quinones may cause oxidative stress in the insect's gut.
3. The absorption of PPO-generated quinones and reactive oxygen species (ROS) produced by redox cycling of quinones could have bacteriocidal and fungicidal toxic effect on insect pests (Yoruk and Marshall 2003).
4. *o*-Quinones polymerize to produce melanin which forms a layer around the infected tissue (Vamos-Vigyazo 1981; Vaughn et al. 1988; Zawistowski et al. 1991).

Though PPOs could potentially be involved in plant defense against pest and pathogen attacks, they are not the only enzymes involved in plant defense. Peroxidase (POD) and its reaction products may mediate in the cross-linking of dietary proteins, thereby reducing the nutritive value (Matheis and Whitaker 1984). POD could be involved in enzymatic browning (synergy with PPO) by utilizing *o*-quinones and H_2O_2, oxidation products of PPOs as its substrates (Forget and Guillard 1997). Phloroglucinol oxidase (PhO), a distinctive PPO with strong POD activity, was characterized from cabbage (Fujita et al. 1995, 1997). The PhO has different active sites for peroxidase and PPO activities. Rahman et al. (2012) have also characterized a single PhO from cauliflower with PPO and POD activities. Further, phenolic compounds generated under stress could directly play a role in plant defense or indirectly through PPO- or POD-mediated reactions (Macheix et al. 1990). Therefore, control of phenylalanine ammonia lyase (PAL) which plays a crucial regulatory role in biosynthesis of phenylpropanoid pathway products (flavonoids, isoflavonoids, hydroxycinnamic acids, coumarins, stilbenes) could prevent undesirable browning in plants (Maritnez and Whitaker 1995; Bagal et al. 2012).

5.2 Role of PPOs in Biosynthesis of Specialized Metabolites

5.2.1 Betalain Biosynthesis

Besides the potential involvement of PPOs in plant defense, they have been implicated in other several roles. Temporal and spatial expression studies using Northern blot analysis in *Phytolacca americana* (pokeweed) indicated the PPO transcripts to be localized specifically to the ripened fruits that had turned red with betacyanin accumulation (Joy et al. 1995). All the tissues in the ripening fruits except the seeds accumulate betalains. Based on this study, it was assumed that PPOs could be involved in betalain biosynthesis. Later, a tyrosinase (PPO) catalyzing L-DOPA (L-3,4-dihydroxyphenylalanine] and *cyclo*-DOPA, two important precursors of betalain biosynthesis, were characterized from betacyanin-producing callus cultures of *Portulaca grandiflora* Hook (Steiner et al. 1999). On further investigation in other betalain-producing plants, the tyrosinase activity was observed only in the plants and red cell cultures of *P. grandiflora* and *Beta vulgaris* L. subsp. *vulgaris* (garden beet group), demonstrating a correlation between PPO activity and betacyanin content.

The PPO could be involved in betalain biosynthesis at different steps including the primary step, conversion of tyrosine to L-DOPA (Fig. 5.2). The L-DOPA could be utilized in producing either yellow-colored betaxanthins or red-colored betacyanin pigments (Fig. 5.2). The L-DOPA is oxidized by PPO to DOPA quinone which can spontaneously generate *cyclo*-DOPA. The *cyclo*-DOPA undergoes condensation with betalamic acid to form the red betacyanin (Gandia-Herrero and Garcia-Carmona 2013). Interestingly, a beet cytochrome P450, CYP76AD1, has been

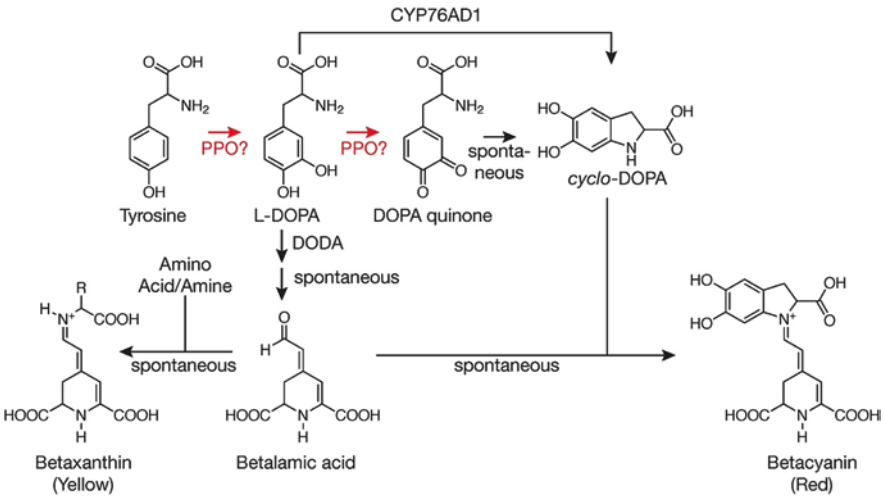

Fig. 5.2 Betalain biosynthesis and probable role of PPOs (Source: Sullivan 2015, with permission)

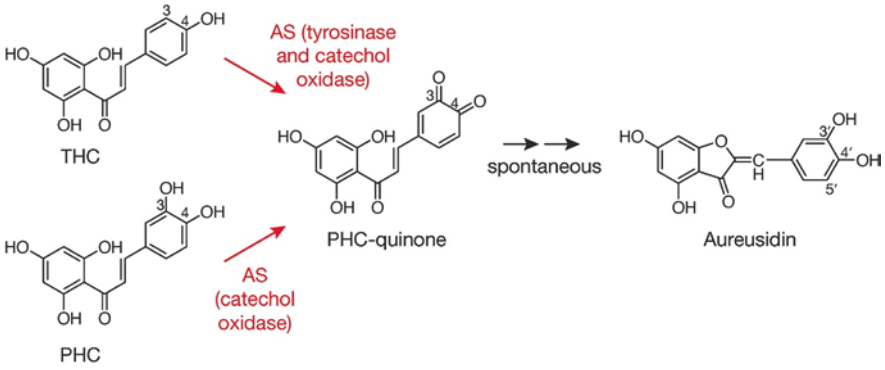

Fig. 5.3 Aureusidin synthase (AS, a vacuolar PPO) involved in the biosynthesis of aurones in *A. majus* (Source: Sullivan 2015, with permission)

identified as a potential enzyme that could be involved in vivo in tyrosine to L-DOPA conversion, DOPA quinone formation, and ultimately producing red betacyanin pigment (Hatlestad et al. 2012). Despite the correlation studies (Steiner et al. 1999; Chang-Quan et al. 2007; Gao et al. 2009) or studying the betaxanthin pathway in tobacco cells using fungal PPO (Nakatsuka et al. 2013), no transcriptomic evidence has been found demonstrating higher expression of PPO which would expect for high betalain production (Hatlestad et al. 2012). Thus, in the assumption that PPOs are involved in betalain biosynthesis, their role in *cyclo*-DOPA production is unlikely, and in vivo demonstration of their role in conversion of tyrosine to L-DOPA is lacking.

5.2.2 Aurone Biosynthesis

PPOs' role in biosynthesis of chalcone-derived yellow-colored aurone pigments in *Antirrhinum majus* (snapdragon) flowers has been demonstrated unambiguously (Nakayama et al. 2001; Fig. 5.3). The glucosides like aureusidin and bracteatin are mainly responsible for yellow color of snapdragon flowers. Aureusidin and bracteatin can be formed from 2′,4′,6′,4-tetrahydroxychalcone (THC) or 2′,4′,6′,3,4-pentahdroxychalcone (PHC) upon incubation with crude flower extracts (Sato et al. 2001).

The enzyme involved in the formation of aureusidin and bracteatin, aureusidin synthase (AmAS1), a monomeric enzyme of 39 kDa, was purified to homogeneity from snapdragon flower buds. Based on the amino acid sequences of peptide fragments, a AS cDNA was isolated and characterized. The amino acid sequence of AmAS1 had high similarity with PPOs from apple (51% identity), grape (47%), and potato (39%; Nakayama et al. 2001). The purified AmAS1 catalyzed both the hydroxylation and cyclization of THC and PHC to produce aureusidin. Interestingly, PHC is a better substrate compared to THC for the purified AmAS1. Also, the aureusidin production from PHC was inhibited by H_2O_2 but was enhanced with

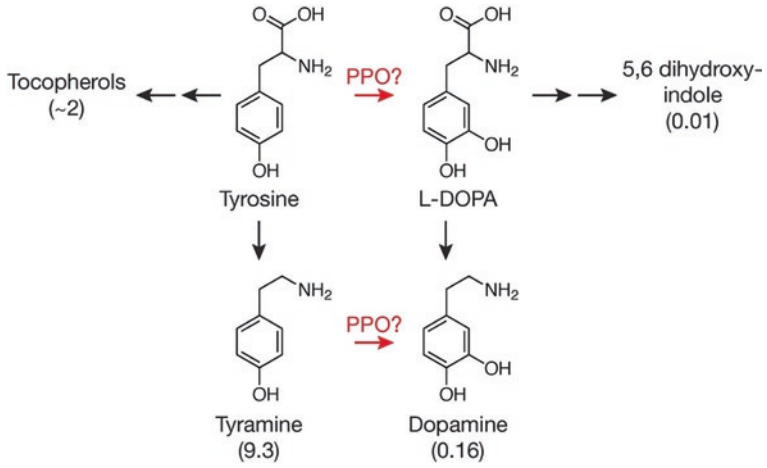

Fig. 5.4 Tyrosine metabolism in walnut as proposed by Araji et al. 2014. The *numbers* in the parenthesis indicate the increase in metabolite in transgenic plant compared to the wild-type plants (Source: Sullivan et al. 2015, with permission)

THC. The conversion of THC to aureusidin by *Neurospora crassa* tyrosinase (related to plant PPOs) showed the ability of other PPOs also to be involved in biosynthesis of metabolites. AmAs1 could not oxidize other monophenols and diphenols like tyrosine, L-DOPA, or caffeic acid, thereby exhibiting a very narrow substrate specificity. Another interesting feature of AmAs1 is its localization to vacuoles instead of plastids (as in case of other plant PPOs; Ono et al. 2006). Gene expression studies showed that AmAs1 is expressed in petals of yellow varieties (aurone containing), and no transcripts were observed in aurone-lacking petals of white, pink, and red varieties. AmAs1 transcripts were not detected in leaf and stem; both the tissues lack AS activity. Kaintz et al. (2014) have identified a plastid-localized and tyrosinase activity lacking AS PPO from *Coreopsis grandiflora* involved in aurone biosynthesis.

5.2.3 Tyrosine Metabolism

The role of PPOs in conversion of tyrosine to L-DOPA has been shown in *Juglans regia* (walnut) by Araji et al. (2014; Fig. 5.4). A walnut PPO having both tyrosinase and catechol oxidase activity with a constitutive expression in herbaceous tissues has been identified and characterized (Escobar et al. 2008; Zekiri et al. 2014). Araji et al. (2014) have studied in vivo PPO function using RNAi transgenic lines that showed negligible catecholase activity (~5% only) compared to the wild-type controls. The transgenic plants developed necrotic lesions on the leaves, but no pathogens were found. Though no differences among the levels of oxidative damage indicators (like H_2O_2) were observed between transgenic and control plants (Lorrain et al. 2003), metabolomic analysis of wild-type and PPO-silenced leaves indicated

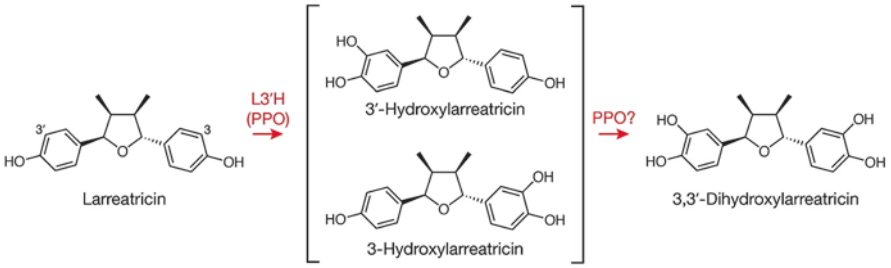

Fig. 5.5 Dihydroxylarreatricin biosynthesis in *Larrea tridentata* (creosote bush). *L3′H* larreatricin 3′-hydroxylase (Source: Sullivan 2015, with permission)

significant differences in levels of several metabolites particularly those associated with tyrosine metabolism (Araji et al. 2014).

The leaves of PPO-silenced plants exhibited enhanced levels of tyramine (~10-fold) and tocopherols (~2-fold) compared to the wild control plants. Interestingly, hydroxylation products of tyrosine and tyramine (substrates of PPO; Fig. 5.4) were reduced in PPO-silenced plants. Dopamine (from tyramine or L-DOPA) and 5,6 dihydroxyindole (from L-DOPA) levels were reduced by ~6- and ~100-fold in PPO-silenced plants compared to the controls. From these results, it was concluded that walnut PPO is responsible for hydroxylation of tyrosine and tyramine (Araji et al. 2014). The appearance of necrotic lesions on leaves of PPO-silenced plants was shown to be due to accumulation of tyramine.

5.2.4 Lignan Biosynthesis

An enantiospecific PPO involved in biosynthesis of linked lignins was isolated and characterized in *Larrea tridentata* (creosote bush; Cho et al. 2003; Fig. 5.5). The creosote bush was widely used as a traditional medicine by indigenous people of Americas for treating different ailments (Train et al. 1941; Waller and Gisvold 1945; Timmermann 1977). Nordihydroguaiaretic acid (NDGA), an antioxidant compound, is the major component of the resinous gum used for treatment (Cho et al. 2003). NDGA and its derivatives have bioactive qualities including anticancer (McDonald et al. 2001), antiviral (Gnabre et al. 1995; Craigo et al. 2000), anti-HIV (Gnabre et al. 1995), and allelopathic properties (Elakovich and Stevens 1985). Cho et al. (2003) identified a protein from creosote bush that was capable of hydroxylating larreatricin to 3′-hydroxylarreatricin. The larreatricin 3′-hydroxylase activity exhibiting protein was purified (~1700-fold), and this enzyme converted only (+)-larreatricin to 3′-dihydroxylarreatricin but not (−)-enantiomer, demonstrating enantiospecific hydroxylation.

The (+)-larreatricin 3′-hydroxylase was isolated from *L. tridentata* twigs and further purified using chromatography. The peptide sequencing data obtained from microcapillary RP-HPLC nano-electrospray tandem MS (μLC/MS/MS) analysis of partially purified enzyme yielded fragments that exhibited high homology to *Vicia*

faba and *Prunus armeniaca* (apricot) PPOs. The creosote bush PPO is a plastid-localized PPO indicating that at least some steps in NDGA biosynthesis could take place in plastids. Though there is compelling evidence to suggest the role of PPO in NDGA biosynthesis, evidence from gene silencing experiments or characterization studies of recombinant protein demonstrating *L. tridentata* PPO's role is still lacking.

5.2.5 Other Roles of PPOs in Plants

PPOs have been implicated in Mehler reaction, photoreduction of O_2 by photosystem I (PSI), and regulation of oxygen levels in plastids (Tolbert 1973; Vaughn and Duke 1984; Vaughn et al. 1988; Trebst and Depka 1995) due to multiple reasons: (i) association of most of the plant PPOs with the plastids, (ii) high K_m (Michaelis constant) of PPO for O_2, and (iii) the induction of PPO and evolution of O_2 in isolated chloroplasts. The Mehler reaction acts as a sink for the unutilized light energy under water-stress conditions when carbon assimilation is reduced due to stomatal closure (Biehler and Fock 1996). The dissipation of excessive energy is very essential as otherwise it is used to form reactive oxygen species ultimately leading to photoinhibition and photooxidative damage (ROS; 44). The plants with enhanced PPO levels would be stress tolerant if PPOs are involved in the Mehler reaction.

The role and response of PPOs under water-stress conditions were examined in tomato plants having different levels of PPO expression (Thipyapong et al. 2004). Surprisingly, transgenic tomato plants with reduced/suppressed PPO (SP) levels showed better water relations (leaf water potential and stomatal conductance), lower chlorophyll (a/b) losses, and decreased/delayed photoinhibition compared to PPO overexpressing (OP) or non-transformed (NT) plants under water-stress conditions. Since SP plants exhibited better performance under water-stress conditions compared to OP or NT plants, it is assumed that plant PPOs may play a role in the development of plant water stress and potential for photoinhibition/photooxidative damage that may not be linked to any effects on the Mehler reaction. Further, among the seven tomato PPOs, only two (PPO B and D) were upregulated in abscission zone of older leaf petioles under water-stress conditions. Additionally, PPO B is also induced in leaf veins and stem vasculature (cortical and pith cells). Interestingly, the pathogen-inducible PPO F was induced by water stress, indicating that induction and downregulation of different PPOs in different plant tissues could be a plant strategy to combat different climatic stresses. Overall, the results from Thipyapong et al. (2004) suggest that PPO may not be involved in Mehler reaction, and if it is, it might not be rate-limiting. Further, enhanced levels of PPO under water stress were earlier reported in tomato (English-Loeb et al. 1997) and coconut (Shivishankar 1988). Therefore, despite some evidence suggesting probable role of PPOs in drought response, its physiological role(s) in water stress response remains unclear and needs to be determined.

Niknam et al. (2006) reported an increase in PPO activity in *Trigonella foenum-graecum* calli at elevated NaCl levels (>100 mM NaCl). The increasing NaCl levels

from 0 to 100 mM did not enhance PPO levels significantly (average ~5.6 ΔA_{430} g^{-1}[f.m] min^{-1}), but at 150-mM NaCl, a substantial increase in PPO levels (26.89 ΔA_{430} g^{-1}[f.m] min^{-1}) was observed. Interestingly, the PPO levels were reduced under UVB treatment of tomato plants probably to reduce the oxidative damage, while the phenolic and anthocyanin content increased (Balakumar et al. 1997). PPOs have been implicated in hydroxylation of *p*-coumaric acid to caffeic acid in the phenylpropanoid pathway (Vaughn and Duke 1984). Lack of significant differences in leaf coumaric acid and caffeic acid pools between PPO-suppressed (SP) and non-transformed (NT) tomato plants suggests no role for PPOs in hydroxylation in leaves (Thipyapong et al. 2004). Tissue localization (Sherman et al. 1991; Sommer et al. 1994), tentoxin treatment of PPO (Vaughn and Duke 1981, 1982) and identification of cytochrome p450 *p*-coumarate 3-hydroxylase (Franke et al. 2002) have shown that PPOs are not involved in coumarate hydroxylation. PPO-inhibited proteolysis of postharvest red clover and alfalfa at the early stage of ensiling is probably through protease inactivation (Sullivan and Hatfield 2006). Increased proteolysis and delayed browning were observed in PPO-suppressed transgenic red clover plants compared to non-transformed controls. But decreased proteolysis coupled with increased browning was observed when PPO was expressed in alfalfa leaf extracts compared to the controls (Sullivan et al. 2004). PPOs have also been suggested to play a role in production of signal molecules, flowering regulation, phenol turnover rate maintenance, and trapping of cadmium crystals, but no conclusive evidence is available for these roles (Ebrahimzadeh and Abrishamchi 2001; Lavid et al. 2001; Goldman et al. 1998; Balakumar et al. 1997).

In nature, PPOs are capable of playing different roles in response to different biotic and abiotic stresses. Correlative studies have suggested different roles for plant PPOs in several species, but they were only indicative in nature. Evidence from antisense/sense technology successfully utilized in functional analysis of PPOs in plant species has demonstrated the role of PPOs in pigment biosynthesis, pest/pathogen resistance, and inhibition of postharvest proteolysis. In the future, this technology should be used in different plant species to understand the other physiological roles of plant PPOs. Several plant species have highly identical multiple PPO genes making the functional analysis of individual members a challenging job. Advanced techniques like RNAi could be utilized in such cases to understand and inhibit the role of specific genes. Additionally, these technologies could be utilized in enhancing the quality of different crop products by downregulation of PPO expression. But since PPOs may be involved in other important functions, the downregulation of genes should be carried out selectively in specific tissues or organs, thereby avoiding appearance of unexpected plant phenotypes. On the contrary, the enhanced expression of PPO may be an efficient and cost-effective strategy to combat pest/pathogen attack, but its effects on water stress physiology and postharvest quality should also be looked into. The best possible available option is to selectively upregulate and downregulate simultaneously the expression in specific organs or tissues depending on the final objective.

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Polyphenol Oxidase(s): Importance in Food Industry

6

Enzymes' role as a catalyst in plant physiology and metabolism is indispensable. Enzymes remain active throughout the life cycle of a crop including at postharvest stages. This is useful in species where the ripening occurs during postharvest storage, but sometimes it leads to deterioration of quality traits including color, taste, texture, and nutritional content. The combination of endogenous deteriorative enzymes and growth of microorganisms can significantly impact the quality of produce, shorten the shelf life, and affect the consumer acceptance of the produce. Despite their influence on quality traits, enzymes are used as biocatalysts in several food-processing applications to hydrolyze large molecules into simpler molecules. Additionally, enzymes are being used to produce and change molecules to improve the organoleptic quality of food products (Terefe et al. 2014). The enzymes responsible for quality losses vary among the different products like PPOs in cereals and fruits (Bhattacharya et al. 1999; Holderbaum 2010), lipoxygenase in certain vegetables, and polygalacturonase in apricots (Luh et al. 1978; Whitaker 1991). According to estimates, PPO is the single most damaging of enzymes in color deterioration of crop products with losses of up to 50% for tropical fruits and vegetables (Whitaker and Lee 1995).

The oxidative enzymes like polyphenol oxidases (PPOs) are responsible for changes in color and flavor in addition to loss of nutritional value (Whitaker 1991; Terefe et al. 2014). These undesirable changes in quality are a major limiting factor in handling and processing of crops since the products undergo rapid browning upon slicing, peeling, and damage due to mechanical reasons or herbivory. Some of the economically important edible products susceptible to undesirable browning include wheat-based products (Bhattacharya et al. 1999); fruits like apple (Holderbaum 2010), banana (Gooding et al. 2001), grape (Rathjen and Robinson 1992), pineapple (Das et al. 1997), and mango (Robinson et al. 1993); and vegetables such as eggplant (Perez-Gilabert and Carmona 2000; Shetty et al. 2011) and potato (Sanchez-Ferrer et al. 1993; Cho and Ahn 1999). Interestingly, the browning reaction enhances the quality of certain products like black tea (Eskin 1990; Ullah

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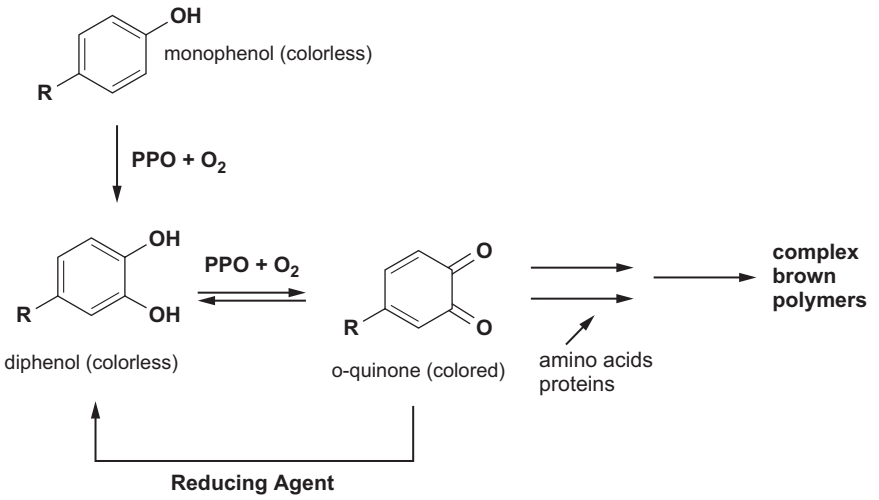

Fig. 6.1 The reaction catalyzed by polyphenol oxidase (Mayer and Harel 1979)

1991), coffee (Amorim and Silva 1968; Amorim and Melo 1991), and cocoa (Lee et al. 1991; Lopez and Dimick 1991).

As mentioned earlier, PPOs catalyze two important reactions: hydroxylation of monophenols to *o*-diphenols and oxidation of *o*-diphenols to *o*-quinones (Tomas Barberan and Espin 2001; Fig. 6.1). The *o*-quinones responsible for enzymatic discoloration of plant products are yellow compounds that are highly unstable and form complexes with amino acids or proteins (Ramaswamy and Riahi 2003). The polymerization of these intermediates and condensation of *o*-quinones produce the characteristic reddish-brown colored polymers called melanins that are responsible for the undesirable browning of food products (Tomas-Barberan and Espin 2001; Ramaswamy and Riahi 2003).

Phenols are the major substrates of PPOs. In food, phenolic compounds contribute to the bitterness, color, flavor, astringency, and oxidative stability of products (Tomas-Barberan and Espin 2001). Interaction between food proteins and oxidized products of phenols leads to covalent condensation resulting in changes in different characteristics of food proteins (Matheis and Whitaker 1984; Yoruk and Marshall 2003). The *o*-quinones interact with the side chains of amino acids, thereby causing a reduction in the nutritive value of food products (Matheis and Whitaker 1984; Felton et al. 1989, 1992). The amino acid side chain groups like –SH and $–NH_2$ are generally susceptible to binding by quinones. A reduction in lysine content was reported in casein and tomato protein in the presence of phenolic compounds and PPOs (Matheis and Whitaker 1984; Felton et al. 1989, 1992). Further, food proteins that have tyrosine or are linked to phenolic groups are modified by PPOs (Matheis and Whitaker 1984). Interestingly, redox cycling of quinones formed during PPO reactions generates free radicals that can damage DNA, amino acid, and proteins (Felton et al. 1992; Hill 1992).

More than 8000 polyphenolic compounds have been identified in various cereals, fruits, and vegetables (Pandey and Rizvi 2009). Cereals, fruits, and vegetables both in their raw and processed forms contain significant amounts of polyphenols. Polyphenols are secondary metabolites that are mostly implicated in plant defense against pathogens. The long-term consumption of polyphenol-rich foods has shown beneficial effects on human health owing to the high antioxidant activity of polyphenols (Heim et al. 2002). Further, several epidemiological studies demonstrated some protection by polyphenols against cancers, diabetes, and neurodegenerative and cardiovascular diseases (Arts and Hollman 2005; Graf et al. 2005). It is interesting that high polyphenol content is helpful for human health, whereas low enzymatic browning is relevant for food processing as to maintain original color, flavor, and nutritional value (Murata et al. 1995; Podsedek et al. 2000). Owing to their economic and nutritional importance, polyphenols and PPOs are a subject of scientific interest for plant breeders, pathologists, food technologists, and medical researchers.

Globally, wheat is the second most important staple food crop with an estimated annual production of 729 million tonnes in 2014 (http://www.fao.org/faostat). Wheat flour is used in preparation of several products including bread, crackers, cookies, biscuits, cakes, noodles, macaroni, and spaghetti. Wheat is mostly consumed in the form of flatbread (Middle East and Asia) and noodles (South and East Asia). Wheat noodles form an important part of the daily diet in several Asian countries, and about ~40 to 50% of wheat flour consumed is used to manufacture different kinds of noodles (Hou and Kruk 1998; Anderson and Morris 2001). The USA, Australia, and Canada are the key suppliers of wheat used in noodle manufacturing in Asia as their wheat is considered to possess desirable quality attributes. Brightness and color (cream/white or yellow) are two very important quality parameters of Asian noodles (Fig. 6.2). Therefore, tailor-made wheat varieties that meet the needs of discriminating buyers, particularly the Asian markets which imports significant quantities of wheat from the USA, Australia, and Canada, need to be developed.

The time-dependent darkening of wheat noodles due to PPOs is critical (Kruger et al. 1994; Baik et al. 1995; Crosbie et al. 1998; Hou and Kruk 1998), more importantly in raw noodles that are stored for several days before cooking (Mares and Campbell 2001). So far, the wheat improvement efforts have focused on developing cultivars with low or reduced PPO content. But even these varieties that contained low or reduced PPO levels possessed certain levels of PPO that caused browning reaction in the wheat products (Fig. 6.2). But collaborative efforts between breeders at Montana State University, Bozeman, USA, and University of Nebraska, Lincoln, USA, have been successful in identifying wheat lines (07OR1074) with negligible or zero PPO activity comparable to durum wheat (Hystad et al. 2015). Breeding efforts are now underway to incorporate these genotypes into wheat breeding programs. The major objective would be to develop varieties with zero/negligible PPO activity in addition to good milling and baking qualities that would be acceptable to the consumers.

The importance of PPOs in plant defense and their involvement in postharvest losses of horticultural crop produce due to enzymatic browning explains why PPOs

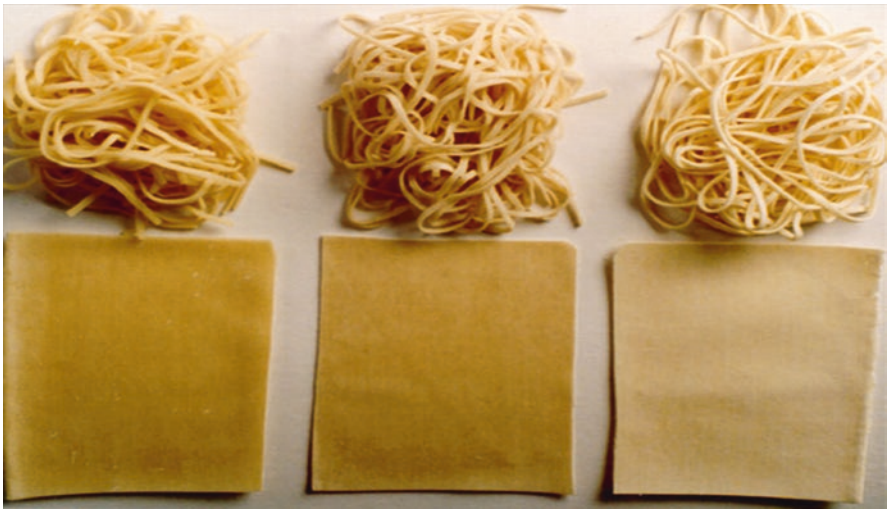

Fig. 6.2 Variation in end-product quality traits in different types of wheat varieties with different PPO levels (Source: Dr. J Martin, Montana State University, Bozeman, USA, with permission)

Fig. 6.3 Tissue prints showing enzymatic browning from eggplant fruits due to mechanical injury (sections **a**–**c**) and shoot/fruit borer infestation (**d**–**f**). Sections **a** and **d,** immunolocalization using immune serum (rabbit anti-GST–SmePPO1); sections **b** and **e,** pre-immune serum; sections **c** and **f,** secondary antibody conjugate (Adapted from Shetty et al. 2011)

have been studied and characterized in several diversified vegetable species (Mayer 2006). Among the crops of Solanaceae family, potato, eggplant (also called brinjal or aubergine), and peppers are the major vegetable crops of substantial economic value (Daunay 2008). Eggplant is an important vegetable crop of Asia; India and China are the leading producers with an annual production of 13.6 and 29.5 m tonnes, respectively, in 2014 (http://www.fao.org/faostat). It is an important ingredient of the Indian cuisine especially for the vegetarians. The PPOs of eggplant (rich in phenols) have been studied extensively due to their role in enzymatic browning of the fruit. Chlorogenic acid is the dominant phenol accounting for ~70 to 95% of total phenols in the flesh of the eggplant fruit (Whitaker and Stommel 2003; Singh et al. 2009). Shetty et al. (2011) have studied and characterized the multigene family of eggplant PPOs. They have demonstrated the browning reaction of eggplant fruit upon mechanical damage and herbivory (Figs. 6.3 and 6.4).

Apple is one of the major fruit crops of the world with an annual global production of ~84.6 million tonnes in 2014 (http://www.fao.org/faostat). The major

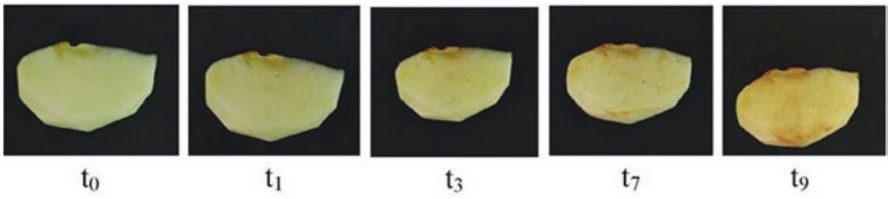

Fig. 6.4 The panel shows a fresh-cut apple slice at zero time (*t0*), after 1 (*t1*), 3 (*t3*), 7 (t7), and 9 (*t9*) days of storage ($T = 7.5$ °C; Adapted from Lunadei et al. 2011)

producers of apples during 2014 are China, USA, Poland, India, and Turkey. The industry consumes about ~40 to 60% of the apples produced, and the rest is sold as fresh produce in the market (Nicolas et al. 1994). Juice, sauce, and slices are the major processed products developed from apples. The spoilage of vegetables/fruits during postharvest manipulations or processing is up to ~25% of the harvested produce (Nicolas et al. 1994). The postharvest losses are estimated to be 30–40% of total production in developing countries (Singh et al. 2014). Postharvest deterioration in apples causes 5–25% losses of total production (Jijakli and Lepoivre 2004). The losses incurred are mostly due to physical injuries, disorders, or diseases and unsuitable conditions for processing. Brown flesh with flattened area on the fruit is a very common defect of apples (Nicolas et al. 1994). Further, browning of the skin or internal tissue is caused due to different conditions including bitter pit, scalds, water core, core flush, and internal breakdown. Improper processing of products (juice, sauce, or slices) could result in discoloration and ultimately reduces the consumer acceptance.

6.1 Control of Browning Reaction

6.1.1 Chemical Control

Browning or discoloration due to PPOs drastically reduces the nutritional and sensory qualities. The PPO-mediated undesirable brown discoloration in cereal products, fruits, and vegetables causes enormous economic impact necessitating its control to reduce the economic losses, maintain product quality, and extend the shelf life of the produce. Therefore, several mechanisms or approaches have been developed to control the undesirable browning. Broadly the available inhibitors could be divided into six classes (McEvily et al. 1992):

1. Reducing agents – ascorbic acid and analogs, sulfites;
2. Chelating agents – ethylenediaminetetraacetate, sodium diethyldithiocarbamate, and sodium azide;
3. Complexing agent – cyclodextrins, chitosan;
4. Acidulants – ascorbic acid, citric acid, malic acid, phosphoric acid;

5. Enzyme inhibitors – substrate analogs, halides; (vi) Enzyme treatments – proteases, and *o*-methyltransferase.

The chemical antibrowning agents eliminate or target different components necessary for the PPO reaction like phenolic substrates and intermediate products like quinones, oxygen, enzyme, or copper molecule (Nicolas et al. 1994; Ahvenainen 1996; Ferrar and Walker 1996; Queiroz et al. 2008). Though different PPOs may have an identical reaction against inhibitors, the inhibitors' effectiveness against different PPOs could vary significantly, thereby necessitating a specific control mechanism for individual PPO systems (Ferrar and Walker 1996).

Reducing agents like sulfites and ascorbic acid are most widespread compounds used in browning control. They control the browning either by preventing the accumulation of *o*-quinones or by forming stable colorless products (Eskin et al. 1971; Nicolas et al. 1994; Osuga et al. 1994; Ashie et al. 1996; Kim et al. 2000). Among the reducing agents, sulfur dioxide and sulfites (sodium sulfite, sodium bisulfite, or sodium metabisulfite) are the most potent inhibitors of browning reaction (Eskin et al. 1971; Sapers 1993; Kim et al. 2000). Sulfites have been used in the vegetable and fruit industry owing to their effectiveness and low price. It is assumed that sulfites directly inhibit PPOs and interact with quinones preventing them from further polymerization (Ashie et al. 1996). But due to adverse health effects of sulfites, the World Health Organization has recommended a limited use or nonuse of sulfites in treatment of fresh fruit and vegetables (Queiroz et al. 2008). Cysteine is another effective sulfur-containing compound used as antibrowning agent. Cysteine reacts with *o*-quinones to produce stable and colorless compounds (İyidoğan and Bayiindirli 2004). Though cysteine is effective in preventing browning at very low concentrations of 1–4 mM (Özoğlu and Bayiindirli 2002; İyidoğan and Bayiindirli 2004), its use in food processing is limited because it produces undesirable odor even at these low levels.

Ascorbic acid (vitamin C) is the best available alternative to sulfites and is often used as antibrowning agent in sliced fruits, canned fruits and vegetables, purees, and fruit juices (Yoruk and Marshall 2003). Ascorbic acid reduces the quinone prior to it undergoing secondary reactions that are responsible for browning and also decreases the pH (Guerrero-Beltrán et al. 2005). The pH optima for PPO reactions are usually in the range of 5–7.5; therefore, lowering of pH inhibits the enzyme activity. The inhibitory effect of ascorbic acid is temporary because it is oxidized irreversibly by reacting with the intermediates like *o*-quinones, endogenous enzymes, metals (e.g., copper), and pigments (Queiroz et al. 2008). But a combination of ascorbic and citric acid was shown to be more effective than ascorbic acid alone (Eskin et al. 1971; Sapers 1993). The acidic environment coupled with the stability of ascorbic acid in an acidic environment could be responsible for increased effectiveness of ascorbic acid. The PPO is inhibited by citric acid through its chelating action and by ascorbic acid via site-directed specificity toward histidine residues of the PPO (Golan-Goldhirsh et al. 1992; Osuga et al. 1994).

The most commonly found acids (like ascorbic, citric, malic, and phosphoric) have been used in food processing to control browning. These acids decrease the pH

to 3 or lower drastically reducing the enzyme activity (Eskin et al. 1971; Park and Luh 1985; Eskin 1990; Osuga et al. 1994). Kojic acid is found in fermented foods as a natural product and has shown antibrowning effect (Queiroz et al. 2008; İyidoğan and Bayiindirli 2004). Koji acid inhibits PPO and also bleaches melanin to colorless compounds. Limited usage of kojic acid in food industry is due to difficulty in large-scale production and higher cost. Aromatic carboxylic acids like benzoic and cinnamic acids inhibit PPO activity owing to their structural similarities with phenolic substrates and by forming a complex with copper (Marshall et al. 2000). β-cyclodextrins inhibit the enzyme activity by probably forming inclusion complexes with PPO substrates (Irwin et al. 1994), and they bind to the substrate's hydrophobic core. The inhibition of enzyme activity is dependent upon the substrate (Casado-Vela et al. 2006) and type of cyclodextrin used (López-Nicolás et al. 2007). Therefore, for effective PPO inhibition, suitable cyclodextrin should be used considering the dominant phenols present in the food product.

A limited number of inhibitors are available that can be used in the food-processing industry due to effective inhibition, undesirable odor/flavor, food safety concerns, and economic feasibility (Eskin et al. 1971; McEvily et al. 1992; Sapers 1993). Honey (Chen et al. 2000), procyanidins (Le Bourvellec et al. 2004), Maillard reaction products (Lee and Park 2005), pineapple juice (Lozano-De-Gonzalez et al. 1993), rhubarb juice (Son et al. 2000a), pectin preparations (Tong et al. 1995), and amino acids with glucose (Tan and Harris 1995) are some of the natural products that have been shown to exhibit a certain inhibitory effect on PPO activity. Honey obtained from different sources decreased the PPO activity by ~2 to 45% in fruit and vegetable products, and enhanced inhibition was observed in combination with ascorbic acid (Chen et al. 2000). Native procyanidins, natural flavanol polymers occurring in plants, inhibited PPO activity probably by forming enzyme–polyphenol–substrate complex (Le Bourvellec et al. 2004). The inhibitory effect of Maillard reaction products produced from amino acids and various sugars depended upon the amino acid [arginine > cysteine > histidine > lysine] and type of sugar [monosaccharides > disaccharides] (Lee and Park 2005).

Proteins, amino acids, and peptides can react with *o*-quinones and chelate the copper at the active site of PPO, thereby affecting PPO activity levels (Queiroz et al. 2008). Girelli et al. (2004) observed that certain glycyl dipeptides like glycylaspartic acid, glycylphenylalanine, glycylglycine, glycyllysine, glycyltyrosine, and glycylhistidine had impacted quinone formation at concentrations ranging from 1 to 50 mM. Different types of inhibition were exhibited by cinnamic acid and its derivatives: cinnamic acid [noncompetitive] > 4-hydroxycinnamic acid [competitive] > 4-methoxycinnamic acid [noncompetitive] (Shi et al. 2005). Cinnamic acid and its derivatives attach to a nonactive site region, thereby either changing the enzyme conformation or hindering the binding of substrate to the enzyme through steric hindrance. Concentration-dependent PPO inhibition was exhibited by several *p*-alkoxybenzoic acids, *p*-methoxybenzoic acid being the most potent inhibitor (Chen et al. 2005). Rhubarb juice and pectin contain oxalic acid, a natural PPO inhibitor that inhibits PPO activity through copper chelation (Son et al. 2000b; Yoruk and

Marshall 2003). Though several natural PPO inhibitors have been identified, they are not yet commercially utilized in the food-processing industry.

6.1.2 Physical Control

The above-described methods of PPO inhibition mostly involve the use of chemical compounds, but several other physical methods like heating, dehydration, irradiation, high pressure, and freezing/refrigeration (Ashie et al. 1996; Kim et al. 2000) have also been used for the control of adverse browning. But some of these methods may have certain disadvantages such as enzyme–substrate contact due to subcellular breakdown and texture deterioration (Macheix et al. 1990). Heat treatment is an effective method to stabilize foods due to its ability to destroy microorganisms and deactivate enzymes. Blanching is most commonly used in vegetables (Marshall et al. 2000), but it is rarely used for treating soft textured fruits or vegetables as it destroys thermosensitive nutrients like vitamins, proteins, and carbohydrates (Lado and Yousef 2002). Large quantities of water and energy requirement coupled with waste disposal problems make blanching technically unattractive. Generally, the catalytic activity of PPO is destroyed in the range of 70–90 °C, but the time needed for deactivation is very variable depending upon the product source (Chutintrasri and Noomhorm 2006). Pineapple PPO activity decreased by ~60% after exposure to 40–60 °C for 30 min, deactivation increased drastically >75 °C, and the residual was 7% and 1.2% at 85 °C and 90 °C for 5 min, respectively (Chutintrasri and Noomhorm 2006).

Good quality and increased shelf life of fruit and vegetable products can be achieved through high hydrostatic pressure (HHP) treatment (Kim et al. 2001). Compared to heat treatment, HHP causes less damage to nutrients like vitamins, pigments, and flavoring agents since HHP has limited or no impact on the covalent bondings in the compounds (Butz et al. 2003). Depending upon applied pressure and its duration, temperature, and protein system (e.g., type of protein, pH, ionic strength), HHP can affect the protein structure and cause protein denaturation or aggregation (Messens et al. 1997). New or analog products can be developed by HHP treatment of foods with no effect on flavor, color, or nutritional content and without any thermal degradation (Vardag and Körner 1995). Further, HHP can modify the functionality of an enzyme, affect the biological activity of the enzyme, and also change its substrate specificity (Hendrickx et al. 1998). The effectiveness of HHP treatment for several PPOs is greater at lower pH and is also influenced by the addition of antibrowning agents, salts, or sugars (Rapeanu et al. 2005). Since PPOs were found to be more resistant to HHP than heat treatment, a combination treatment of high pressure (>400 MPa) and mild heat treatment (~50 °C) was found to be best to inactivate the enzyme in banana puree (*Musa* sp.; Palou et al. 1999), lychee (*Litchi chinensis*; Phunchaisri and Apichartsrangkoon 2005), and strawberry (*Fragaria* × *ananassa*; Dalmadi et al. 2006). Interestingly, low pressure (≤400 MPa) also induced PPO activation in red raspberry (*Rubus idaeus*; Garcia-Palazon et al. 2004) and pear (Asaka and Hayashi 1991) and in apple juice (Anese et al. 1995).

Therefore, the level of pressure applied for inactivation of PPO activity is very critical.

Irradiation with gamma (γ) rays is a physical treatment involving direct exposure of fruit and vegetable products for food preservation, extending their shelf life, and improving their safety and quality (Lacroix and Ouattara 2000). Additionally, radiation treatment destroys the bacteria and fungi, guaranteeing total disinfection, and also delays the ripening process (Iemma et al. 1999). PPO activity in irradiated (1.0 kGy) fresh-cut lettuce was ~31% lower compared to untreated sample, but after 9 days PPO activity of the irradiated samples was ~54% higher than in the control sample (Zhang et al. 2006). Similarly, in fresh-cut celery at 1 kGy (0.5 kGy/h) treatment, the PPO activity was reduced by 73% in the treated sample, and after 9 days the PPO activity was only ~25% lower than in the control sample (Lu et al. 2005).

Pulsed electric field (PEF) is a nonthermal food preservation technology that is mostly focused to inactivate microorganisms and is used in processing of liquid foods (García et al. 2003, 2005; Evrendilek et al. 2004; Li and Zhang 2004). Electric field strength, time of PEF treatment (Zhong et al. 2007), electrical conductivity, and pH affect the inactivation of PPO. The average electric field strength of ~25 kV/min given for 744–6000 μs decreased the PPO activity by 70–97% in different fruit products (Zhong et al. 2007; Giner et al. 2001, 2002). The sensitivity of different enzymes to PEF treatment varied as pepsin > PPO > peroxidase > chymotrypsin and lysozyme (Yang et al. 2004). PEF treatment of PPO results in the loss of α-helix fractions and induces changes in its secondary structure (Zhong et al. 2007). Other technologies used for reducing PPO activity include supercritical carbon dioxide and ohmic and microwave heating (Queiroz et al. 2008). Supercritical carbon dioxide is a nonthermal treatment that physically destroys the microbial cells (Corwin and Shellhammer 2002) and induces enzyme inactivation by causing changes in the secondary and tertiary structure of the enzyme (Gui et al. 2007). Reduced PPO activity was observed when food products were treated with supercritical carbon dioxide (Gui et al. 2007) or in combination with HHP (Corwin and Shellhammer 2002).

PPO is an important enzyme in the food-processing industry, and its activity, in general, causes undesirable discoloration (browning reaction) of food products. The browning of food products decreases their nutritional quality, lowers consumer acceptance levels, and causes economic losses. Therefore, identification of safe antibrowning agents or developing improved technologies to control browning is very critical to enhance product value and minimize the economic losses. Though several technologies have been demonstrated, only a few like HHP have been adopted in food-processing industries. The knowledge about these technologies and the combination of technologies to be used to obtain a high-quality product is very essential.

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Advances in Polyphenol Oxidase (PPO) Research

7

The polyphenol oxidase (PPO) gene family has been extensively studied in Poaceae, Solanaceae, and other plant species in which the PPO activity impacts the economically important aspects like quality and disease resistance. As discussed earlier, plant PPOs exhibit large variability in their size, structure probably due to lineage-specific expansion, and gene loss (Tran et al. 2012). Surprisingly, no PPO genes have been reported in certain species like *Arabidopsis thaliana*. Though majority of plant PPOs show certain conserved features including dicopper center with six ligating histidine residues, N-terminal transit peptide and a C-terminal region, rare variants have also been reported (Tran et al. 2012). Below, latest developments in PPO research involving genetics, genomics, microRNAs, mutagenesis, and others are discussed.

7.1 Genetic and Genomic Aspects of PPOs

Among the different species in which PPOs have been identified and characterized, wheat (both durum and hexaploid) PPOs have been extensively studied. Jukanti et al. (2004) suggested the presence of at least six PPO genes in hexaploid wheat, clustering into two with three similar sequences each. Further, it was speculated that each of the hexaploid wheat genomes (A, B, D) contributed at least two PPO genes (Jukanti et al. 2006). Wheat PPOs were mostly mapped on to the group 2 homoeologous chromosomes by several workers, but PPO loci were also located on group 3, 5, 6, and 7 chromosomes (Jimenez and Dubcovsky 1999; Demeke and Morris 2002; Taranton et al. 2017). Jimenez and Dubcovsky (1999) used cytogenetic stocks (hexaploid substitution and tetraploid substitution lines) to identify the chromosomal location of PPOs in common and durum wheat. Substitution lines of "Cheyenne," "Timstein," and "Thatcher" chromosome in "Chinese Spring" indicated that chromosome 2A is one of the major sources of high PPO activity in hexaploid wheat. Similarly, genes located on homoeologous group 2 chromosomes were linked to higher PPO activity of "Chinese Spring" (2D) and *T. turgidum* var.

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dicoccoides (2A) relative to *T. turgidum* var. *durum* cv. "Langdon." The results indicate that PPO actitivity was due to genes present distally on the long arm of homoeologous group 2 chromosomes.

Later, a double haploid (DH) population (Zhongyou 9507 X CA 9632) of 71 lines was used to construct a linkage map (2881 cM and 21 linkage groups) and in QTL mapping studies (Zhang et al. 2005). Further, two putative major QTLs were identified on chromosome 2AL and 2DL accounting for 37.9–50.0% and 25–29.1% of the phenotypic variance, respectively. The QTL on chromosome 2AL was linked to a simple sequence repeat (SSR) marker *Xgwm*294. Further, Watanabe et al. (2004) also mapped two genes responsible for high phenol color reaction of wheat kernels to the long arms of chromosome 2A and 2B. A DH population of 163 lines derived from Sunco × Tasman, Australian wheat varieties, was utilized to map components of flour and noodle color (Mares and Cambell 2001). A major QTL identified on 2DL (accounted for 23% phenotypic variance) was associated with grain PPO activity and affected noodle brightness/color. Six QTLs were identified for PPO activity from a recombinant inbred line (RIL) population of Louise X Penawawa (Beecher et al. 2012). Four QTLs were located on chromosome 2B and two on 2D chromosome. The fourth QTL on 2B corresponded to *Ppo-B2*; this result is interesting given that Beecher and Skinner (2011) did not detect the expression of *Ppo-B2*. In this RIL population, the *Ppo-B2c* allele of Louise was associated with lower PPO activity than *Ppo-B2a* allele donated by Penawawa. The presence of missense mutation downstream of the first copper-binding region in *Ppo-B2c* allele may negatively impact the PPO activity. Further, another QTL identified on 2B flanked by SNP markers *Xiwa* 905 and *Xiwa* 3908 corresponds to a previously reported QTL in this population. QTL for stripe rust resistance, kernel hardness, and softness equivalent was identified at the same locus by Carter et al. (2009, 2011), and Smith et al. (2011), respectively.

Several studies indicated that the PPO genes located on the homoeologous group 2 chromosome are arranged into two paralogous families, *Ppo-1* [*Ppo-A1*, *Ppo-B1*, and *Ppo-D1*] and *Ppo-2* [*Ppo-A2*, *Ppo-B2*, and *Ppo-D2*] (Jukanti et al. 2004; Beecher and Skinner 2011; Beecher et al. 2012; Martin et al. 2011; Taranto et al. 2015). Among these genes, two kernel types (*Ppo-A1 and Ppo-D1*) have been genetically characterized from bread wheat, and chromosomal location is identified (Sun et al. 2005; He et al. 2007). The location of *Ppo-A2* group of genes is 8.9, 11.4, and 10.7 cM proximal to the respective paralogs of *Ppo-A1*. *Ppo-A1* and *Ppo-D1* allelic variation explained 80% and 2% of the variation in kernel PPO activity, respectively (Martin et al. 2011). Further, functional markers were developed for PPO genes in common wheat. In silico cloning and experimental validation were utilized to characterize two PPO genes, each located on chromosomes 2A and 2D along with their allelic variants (He et al. 2007). Two haplotypes on chromosome 2A showed very high level of sequence similarity (99.6%) at DNA level compared to those on chromosome 2D (95.2%). Two STS markers, PPO16 and PPO29, developed based on the haplotypes located on chromosome 2D which amplified a 713-bp and 490-bp fragments in cultivars with low and high PPO activity, respectively. Using a doubled haploid population from Zhongyou 9507 × CA9632 and a set of

nullisomic-tetrasomic lines and ditelosomic line 2DS of Chinese Spring, the two markers (PPO-16 and PPO-29) were mapped on chromosome 2DL. Additionally, a multiplexed marker combination of PPO33/PPO16 was developed to simultaneously detect the loci on 2A and 2D. The STS marker PPO33 for the PPO gene on chromosome 2A amplifies a 481-bp and a 290-bp fragment from cultivars with low and high PPO activity, respectively.

In the *Ppo-1 and Ppo-2 gene families*, the location of intron I is conserved both within and among the two families (Beecher and Skinner 2011). Similarly, the intron II is conserved between *Ppo-1* and *Ppo-2* groups but is absent in *Ppo-2Aa*, *Ppo-A2b*, and *Ppo-D1a*. Based on comparison of *Ppo*-1 group genes, it is assumed that the intron II might have been present in the ancestral kernel-specific sequence but was probably lost in A or its diploid progenitor *Triticum urartu*. The intron II also appears to have been lost in the D genome that gave rise to *Ppo-D2a* but was retained in progenitor of *Ppo-D2b* (Beecher and Skinner 2011). Intron II fits the ideal criteria for loss as suggested by Roy and Gilbert (2005). Based on a study of intron loss across seven sequenced genomes, they concluded that introns present at the 3′ position which does not interrupt a codon (i.e., phase zero position) are preferentially lost. The intron II of *Ppo-2* family fits the proposed criteria of Roy and Gilbert (2005). Interestingly, the literature does not indicate any significant role of chromosome 2B in PPO activity, although it is mentioned in some reports (Demeke et al. 2001; Fuerst et al. 2008; Watanabe et al. 2004). Finally, it is obvious that *Ppo-1* and *Ppo-2* genes are involved in kernel PPO gene expression, are located on the same chromosomal arms, and most probably are linked. Further, this would be in agreement with the proposal that the two paralogs arose as a gene duplication event by Jukanti et al. (2004). Genetic linkage of both the paralogs if indeed is true, this would have significant implications for wheat breeding efforts that try to minimize the PPO activity levels in cultivars.

Two barley PPO homologs (*PPO1* and *PPO2*) were amplified using a primer pair designed in the wheat PPO copper-binding domains, and the respective full-length PPO genes were cloned using a BAC library, inverse PCR, and 3′RACE (Taketa et al. 2010). Among the majority of the tested accessions, amino acid substitutions of five types affecting functionally key motif(s) or C-terminal region were identified in *PPO1* (Fig. 7.1). But in *PPO2* only one mutant allele with an earlier than expected stop codon due to an 8 bp insertion in the first exon was found in three accessions (Fig. 7.1). Overall, seven alleles in *PPO1* and four alleles in *PPO2* were identified. In *PPO1*, two phenol reaction-positive accessions (HA2 and FI1) had identical sequence, but another positive accession J239 had an identical sequence to two phenol reaction-negative accessions (C2-60 and C1-76). The remaining five phenol reaction-negative accessions (I341, I677, I750, K095, and U004) had nucleotide modifications (described above) that affect their functionality. The results (including RT-PCR analysis) clearly indicate that *PPO1* is the major player controlling the phenol reaction of hulls, caryopses, and rachises; its expression is maximum at 2–3 weeks after flowering. Further, *PPO2* controls the phenol reaction in the crease on the ventral side of caryopses, and its expression peaks at 2 weeks after flowering. The differential expression of the two PPO genes in barley could be due to an

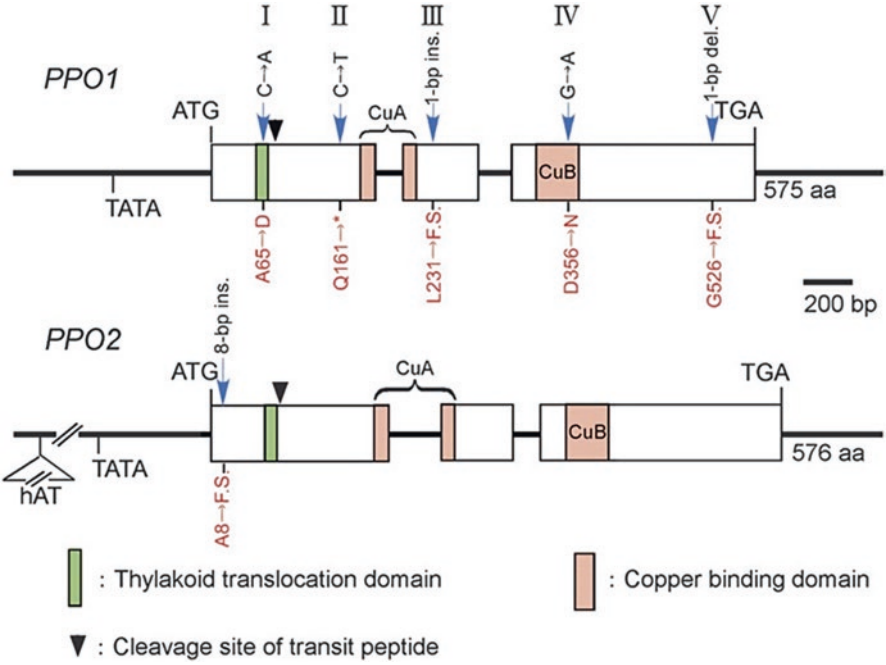

Fig. 7.1 Structure of the PPO1 and PPO2 genes in barley. *Boxes* show exons; the *black bar* between the *boxes* shows introns. *Blue arrows* indicate critical mutation sites that were detected in phenol reaction-negative accessions (types I–V). The *asterisk* denotes a stop codon, and F.S. signifies a frame shift. In the proximity of the promoter region of the PPO2 gene, an insertion of a hAT-family transposon was found (Source: Taketa et al. 2010, J Exp Bot 61(14): 3983–3993)

insertion of a hAT-family transposon in the promoter region (Fig. 7.1). The transposon insertion in the promoter region has been shown to modify the gene expression (Lee et al. 2009; Hayashi and Yoshida 2009). Both *PPO1* and *PPO2* were located on the long arm of chromosome 2H using wheat–barley chromosome addition lines and molecular markers.

The two subspecies of rice differ widely [in >40 characters] (Kato et al. 1928; Oka 1958), but usually the *indica*- and *japonica*-type cultivars are distinguished by four traits, cold tolerance, hair length on glume tips, phenol reaction [PHR], and resistance to $KClO_3$ (Oka 1953; Morishima and Oka 1960). PHR is of particular interest as it reflects grain reaction to phenol treatment (Oka 1953; Morishima and Oka 1960). The grains of *indica*-type cultivars turn brown after being soaked in phenol solution, i.e., positive PHR, whereas in *japonica*-type cultivars the color does not change, PHR-negative (Oka 1953). Yu et al. (2008) characterized *Phr1* that codes for a polyphenol oxidase. Earlier studies have shown that a single Mendelian gene (*Phr1*) controls phenol reaction, and it was shown that *Phr1* is located on the long arm of chromosome 4 (McCouch et al. 1988; Saito et al. 1991; Tanksley et al. 1993; Lin et al. 1994). Based on the mapping results, two PCR-based markers, *S100* and *S115*, present on either sides of *Phr1* in linkage map were developed (Chen

et al. 2002). Fine-mapping efforts pinpointed the location of *Phr1* locus to an 88 kb interval between *P80* and *P168*. Among the 14 open reading frames (ORFs) present within this fragment, *OSJN-Ba0053K19.18* (referred to as K18) was highly similar to plant PPOs (Cary et al. 1992; Chevalier et al. 1999; Constabel et al. 2000; Gooding et al. 2001; Demeke and Morris 2002). The K18 sequence was amplified from MH63 (*indica*-type; high PPO activity) and CJ06 (*japonica*-type; PPO activity undetectable). The sequence comparison showed an 18-bp deletion (Δ18) in CJ06 ORF and also in Nipponbare (japonica type; PHR-negative) compared to MH63, strongly suggesting that K18 is indeed *Phr1* gene (Yu et al. 2008). A complementation test showed that transgenic Nipponbare (PHR-negative reaction) having the entire *Phr1* gene was rescued, whereas the control (with truncated *Phr1*) failed. These results demonstrate that the K18 is Phr1, and the Δ18 is responsible for the loss-of-function phenotype of the japonica-type Nipponbare. Further, *Phr1* demonstrated high sequence similarity with LOC-Os04g53300, codes for polyphenol oxidase with 570 amino acid residues. Interestingly, the *Phr1* contains two introns, two putative copper-binding sites, and a thylakoid-targeting sequence at its N-terminal, rich in hydroxyl amino acids as in other plant PPOs (Constabel et al. 2000).

Tomato (*Solanum lycopersicum*) PPO gene family consists of seven members (A, A′, B, C, D, E, and F) that form three structural classes (I, II, and III) based on restriction fragment length polymorphisms [RFLP] (Newman et al. 1993). The seven genes are clustered within a 165 kb locus on chromosome 8 (Newman et al. 1993). Phage insert mapping showed that PPO A, B, D (classes I, I, II), and E/F (both III) are clustered within separate 12.4 kb clusters. Among the seven tomato PPO genes, PPO B and E/F transcripts were the most abundant, especially in young leaves and inflorescences (Thipyapong et al. 1997). None of the seven PPO genes had introns. Five of the seven PPOs had divergent DNA sequences at their 5′ promoter regions. Newman et al. (2011) studied the temporal and spatial expression of PPO B during tomato development. The PPO B promoter along with the putative *cis*-acting was sequenced and subjected to homology analysis. Most sequence matches were to the 5′ or 3′ flanking DNA sequences or introns of gene encoding proteins involved in pathways/systems like phenylpropanoid biosynthesis, signal transduction pathways, hormonal or stress response, seed and fruit proteins, and photosynthesis (Newman et al. 2011).

Differential expression of PPO B was observed in both vegetative and reproductive tissues. Mostly, PPO B expression was reported to be highest in young tissues and decreased with age. In most organs, PPO B expression was found to be highest in young tissues and decreased with age. Further, the expression was highest in vascular tissues and abscission zones in most tissues. PPO B expression was often found in spots in a specified tissue, typical of enzymes in general phenylpropanoid metabolism (Hahlbrock and Scheel 1989). Staining was observed on all epidermal and internal tissues of petioles that led to leaflets and floral buds; additionally, major veins of apical leaves had predominantly internal tissue expression (Newman et al. 2011). The expression of PPO B in all leafy tissues decreased in older leaves. PPO B expression was observed in stems, particularly in xylem, xylem parenchyma, pith tissues, and cells in vascular bundles. A lower level of expression was observed on

cambium, cortex, and phloem tissues. No expression was observed in tomato roots. At anthesis, the expression was observed in speals, petals, pollen, ovary attachment area, anther attachment area, petal attachment area, pedicels, and pedicel joints. But after anthesis the expression decreased and was observed only in pollen, petal attachment area, ovary attachment area, and pedicel joints. The expression was also observed in stigma/style junction, style/ovary junction, and internal style tissues. Simultaneous expression in all areas of developing fruits was observed. The expression was clearly observed in the outer half of the pericarp, developing seed coats of ovules, vascular tissues, placental tissues, and pedicel joints. The patterns and intensity of staining of tissues remained almost the same between the 5 mm and full-sized green fruit stages. Based on the results, it seems like ethylene regulates the PPO B expression.

Thipyapong and Steffens (1997) studied the inducibility of the tomato PPO gene family (through RNA analysis) following injury. Mechanical injury or infection (*Alternaria solani*) of leaflets at node 5 resulted in systemic induction of only PPO E/F transcripts at leaf nodes 1–3. Mechanical injury at leaf nodes 3–6 led to the induction of only PPO F construct; it showed significantly increased promoter activity. The PPO F promoter construct was induced upon by both abiotic injury and biotic injuries in young leaves (nodes 1–3) following both bacterial (*Pseudomonas syringae*) and fungal (*A. solani*) infection at 72-h post inoculation. Distinctly, cell- and developmental stage-specific patterns of PPO F expression were observed upon different injuries, salicylic acid, ethylene, and jasmonate treatments. PPO F was induced only in young leaves' nodes 1–3 by jasmonates and mechanical wounding and ethylene-induced PPO F only in older leaves (node 7). Interestingly, salicylic acid caused an induction of PPO F at all developmental stages in stems and leaves. A section of ~6 kb in the PPO F 5′ flanking region is sufficient to induce the gene in response to different stresses and wide range signaling molecules. But how induction occurs either through a series of distinct *cis*-elements or a single common *cis*-element present in the 5′ flanking sequence is not yet determined.

The potato (*Solanum tuberosum*) gene family consisting of five PPOs (*POTP1, POTP2, POT32, POT33,* and *POT72*) was identified and characterized (Thygesen et al. 1995; Hunt et al. 1993). POTP1 and POTP2 had >97% sequence identical at DNA level. Similarly, 70–82% sequence similarity was observed between POTP1/P2, POT32, POT33, and POT72. Expression analysis revealed that POTP1 and POTP2 genes are expressed mainly in leaves and flowers, POT72 in roots, and POT32 and POT33 were mostly detected in tubers, POT32 being the major gene during tuber development (Thygesen et al. 1995). The interspecific potato/tomato PPO sequence pairs of POT32/PPO D, POT33/PPO B, and PPO-P2/PPO E exhibited significantly high (>92%) identity at protein level. Recently, a potato genome database survey by Chi et al. (2014) identified nine PPO-like gene models, denoted to as *StuPPO1-9*. The *StuPPO1* to *StuPPO8* are located on chromosome 8, whereas *StuPPO9* is aligned on chromosome 2. *StuPPO1* and *StuPPO6* are closely associated in a 47 kb region, while *StuPPO2-StuPPO5, StuPPO7,* and *StuPPO8* are arranged in tandem in a 144 kb region on chromosome 8. The two regions are separated by a distance of 2072 kb.

Chi et al. (2014) suggested based on high nucleotide sequence similarity (95–99%) that *StuPPO1* to *StuPPO4* could potentially be allelic variants to *POTP1/POTP2, POTP32, POTP33,* and *POTP72*. The expression levels of *StuPPO5, StuPPO6,* and *StuPPO7* might be very low considering their low prevelance in EST databases. Further, *StuPPO8* and *StuPPO9* are the only gene models with no introns. Interestingly, no EST from potato EST databases was found for *StuPPO8*, indicating that this gene might not be transcribed. The PPO activity was very high in roots, stolons, tuber buds, and undeveloped flowers (Thygesen et al. 1995). But, young leaves, mature tubers, and opened flowers exhibited moderate levels of PPO activity. Low PPO activity was present in mature leaves and stems. The contribution of nine PPO-like gene models (*StuPPO1* to *StuPPO9*) to the total PPO protein content was not equal; StuPPO1 (POTP1/P2), StuPPO2 (POT32), StuPPO3 (POT33), and StuPPO4 (POT72) were largely responsible for the total PPO protein content. The suppression or expression of one or some of the PPO genes did not modify the expression of the other genes, but complete non-browning phenotypes with maximum reduction in PPO activity require simultaneous suppression of the expression of *StuPPO1 to StuPPO4* genes.

Six distinct eggplant PPO genes (*SmePPO1* to *SmePPO6*) were identified and characterized (Shetty et al. 2011). The pair-wise comparison of genome and cDNA sequences of the six PPOs indicated the absence of introns, in agreement with the dicotylednous PPOs (Cary et al. 1992; Newman et al. 1993; Sullivan et al. 2004). The sequence homology of *SmePPO1* to *SmePPO3* at nucleotide and amino acid level was 80% and 70%, respectively. Similarly, *SmePPO4* to *SmePPO6* exhibited 89% at nucleotide and 80% identity at protein level. This distinction based on similarity levels indicates the existence of two structural classes of eggplant PPOs. The six eggplant PPOs contain chloroplast transit peptide with conserved cleavage sites for stromal processing [VSCK/N;] and thylakoid processing [L(A/T)A(S/N)A] peptidases with obvious deletions or substitutions in the flanking regions. The C-terminal domain shows highly conserved signature motif (KFDV) resembling the C-terminal of hemocyanin (Marusek et al. 2006). Three *N*-glycosylation sites have been predicted in all the eggplant PPOs except *SmePPO2*. In addition to the well-conserved domain -Asn-Leu-Thr- (-NLT-) shared by all glycosylated PPOs, other *N*-glycosylation sequences have been predicted in *SmePPO1* (-Asn-Gly-Thr-; Asn-Thr-Ser-), *SmePPO3* (-Asn-Gly-Thr-; -Asn-Ala-Ser-), and *SmePPO6* (-Asn-Trp-Thr-). Based on homology-based elucidation of genomic organization of the six eggplant PPOs genes and phylogenetic analyses, it appears that the eggplant PPOs are present as a cluster on chromosome 8, similar to tomato and potato PPOs. The mapping of PPO genes on the chromosome 8 of these three species suggests an evolution based on tandem duplication events.

The transcript levels of the six eggplant PPO genes differ significantly in different plant tissues. While *SmePPO1* gene was expressed in all the plant tissues analyzed, *SmePPO2* was expressed predominantly in root tissues. The expression of *SmePPO3* and *SmePPO4* was observed in all the tissues except the roots. The co-expression of *SmePPO1* and *SmePPO3* was observed in fruits and at all the developmental stages of flowers. But, only moderate expression of *SmePPO5* and

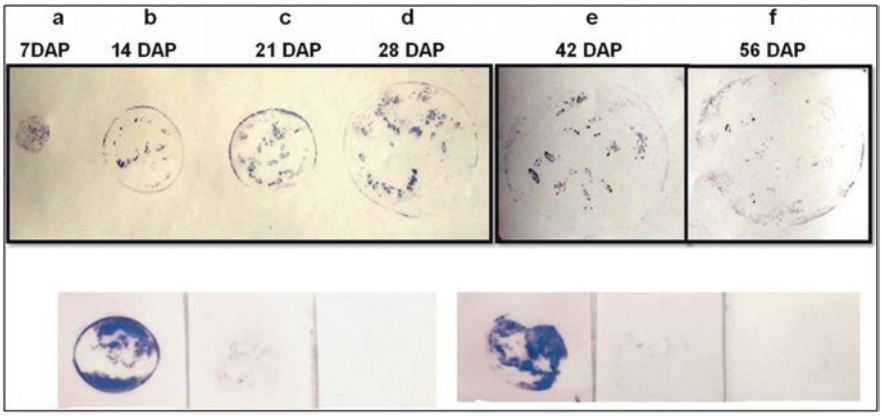

Fig. 7.2 Immunocytolocalization of PPO in eggplant fruit at different stages of fruit development. Tissue prints from different stages of eggplant fruit as indicated herein: sections (**a**–**f**) represent 7 DAP, 14 DAP, 21 DAP, 28 DAP, 42 DAP, and 56 DAP, respectively. DAP, days after pollination (*Source*: Shetty et al. 2011, Phytochem 72: 2275-2287, with permission)

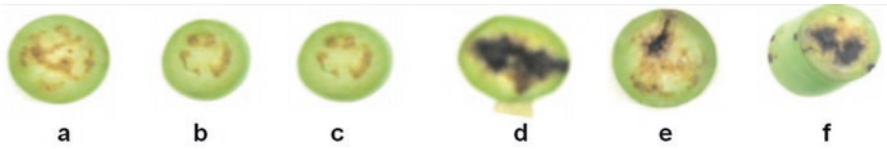

Fig. 7.3 Tissue prints from eggplant fruits following mechanical injury (sections **a**–**c**). The eggplant fruit slices shown here are at 10 min following slicing and indicates enzymatic browning. Tissue prints from eggplant fruits following shoot and fruit borer infestation (sections **d**–**f**). Immunolocalization using immune serum (rabbit anti-GST–*SmePPO1*) is shown in sections **a** and **d**, and those with pre-immune serum are shown in sections **b** and **e**. Tissue prints using only secondary antibody conjugate are shown in sections **c** and **f** (Source: Shetty et al. 2011, Phytochem 72: 2275–2287, with permission)

SmePPO6 was reported in young leaves, in buds, and in fruits. Interestingly, PPOs of Solanaceae that share sequence similarity appear to demonstrate tissue-specific expression patterns. The two eggplant PPOs (*SmePPO1* and *SmePPO2*) are consistently expressed in roots similar to tomato PPOs A, C, D, and potato PPO32 allele. Similarly, *SmePPO1* shows comparable expression as tomato PPOs B and D. The tissue printing studies demonstrated the presence of PPOs predominantly in exocarp (peel), region surrounding the seeds, and was absent in major part of mesocarp (pulp) of the eggplant fruit (Fig. 7.2).

The eggplant fruits subjected to wounding or shoot and fruit borer infestation exhibited PPO localization mostly around the site of wounding or infestation (Fig. 7.3). The localization of PPO around the site of infestation is responsible for the brown/black pigmentation. Thus, it appears that the eggplant PPOs are upregulated or activated by mechanical wounding or pest attack. The browning reaction seems to occur due to tissue injury in both the cases. All the eggplant PPOs except

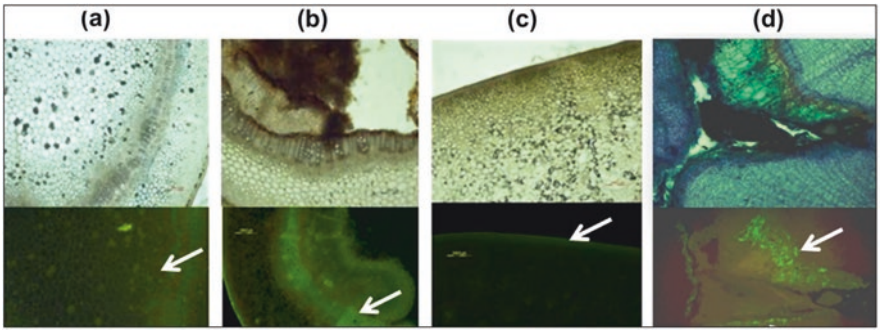

Fig. 7.4 Localization of PPO by immunofluorescence in eggplant infected with shoot and fruit borer. Primary antibody: rabbit antiserum to GST-eggplant PPO1 (1:10,000 dilution); secondary antibody: goat anti-rabbit IgG-FITC conjugate (1:2500 dilution). Panels (**a**) and (**b**) Normal eggplant stem and SFB-infested stem, respectively. Panels (**c**) and (**d**) normal eggplant fruit and SFB-infested fruit, respectively. Unit scale indicated in all the panels represents 250 lm. The *top row* indicates bright field images, and the *bottom row* indicates corresponding fluorescent images. *White arrows* in panels **a**–**d** indicate the immunofluorescence from PPO localized in SFB-infested eggplant tissues (Source: Shetty et al. 2011, Phytochem 72: 2275–2287, with permission)

SmePPO2 were induced and upregulated following mechanical injury in the fruit over 6-h period, but *SmePPO2* and *SmePPO3* transcripts were undetectable in the stem. The expression of *SmePPO1* and *SmePPO4* to *SmePPO6* was most prominent over a 2-h period. The PPO localization using immunofluorescence confirmed the localization of PPO around the site of shoot and fruit borer infection in tender stems and fruits (Fig. 7.4) in comparison to the normal eggplant fruit.

In banana four PPO cDNA sequences (BPO1, BPO11, BPO34, and BPO35) were amplified from banana fruit (Gooding et al. 2001). The BPO1 cDNA was the most abundant sequence, and its genomic sequence contained a 85-bp intron. High levels of PPO activity were observed in banana flesh throughout growth and ripening. Wounding or treatment with 5-methyl jasmonate did not induce PPO activity significantly in banana fruit. Expression analysis detected BPO1 and BPO11 in banana flesh, while BPO34 and BPO35 were undetected in developing fruits. Further, BPO1 and BPO11 (very low levels) were also detected in flowers, stem, roots, and leaf roll samples but was undetected in mature leaves. BPO34 and BPO35 were detected at a low level in flowers and roots, while BPO34 was the only transcript detected in mature leaves. Recently, an artichoke (*Cynara cardunculus* var. *scolymus* L.) [*CsPPO*] PPO gene was characterized (Quarta et al. 2013), and *Cis*-acting elements were identified in the promoter region, probably involved in light and wound response. Significant induction of *CsPPO* was observed 48 h after the wounding of capitula though browning reaction started earlier. Esccobar et al. (2008) identified a PPO [*JrPPO1*] in walnut (*Juglans regia*) using southern analyses, later genome sequencing efforts in walnut identified as second PPO homolog [*JrPPO2*] (Martínez-García et al. 2016). *JrPPO1* and *JrPPO2* shared sequence identity of 80% and 71% at nucleotide and amino acid level, respectively. *JrPPO1* and *JrPPO2* are differentially expressed, with *JrPPO1* mostly expressed in green

tissues (leaves, pistillate flower, hulls) and *JrPPO2* specifically in callus tissue, where expression of *JrPPO1* is lower.

7.2 MicroRNA (miRNA) Technology in PPO

MicroRNAs are small (21 nucleotide in length), noncoding, and endogenous RNAs that are found in plants, animals, and in some viruses (Bartel 2004; Chen 2009; Karlova et al. 2013). They play critical roles in plant development through gene expression regulation by degrading mRNA or translation control (Bartel 2004; Bushati and Cohen 2007). The cleavage site in plants is between the 10th and 11th nucleotide from the 5′ end of the miRNA in the mRNA-miRNA duplex (Rhoades et al. 2002). The cleavage results in a 3′ fragment of the target mRNA having a monophosphate at its 5′ end, a key factor that validates a miRNA target (Llave et al. 2002). The miRNAs are involved in RNA silencing pathways and induce silencing of the gene of interest (Sablok et al. 2011). The major advantage of a miRNA technology is that it produces identical small RNA population with similar selective sequence. It is a viable method for either silencing an individual gene or a multigene family with the minimum risk of affecting unpredicted off-target (Schwab and Voinnet 2010). Due to rapid advances in high-throughput sequencing technology, several miRNAs along with their target genes have been reported in several plant species (Chen 2012). The miRNA is relatively a new technology that has been largely used for inducing loss of gene function in plants (Alvarez et al. 2006; Schwab et al. 2006; Tang et al. 2010; Zhao et al. 2009). The identification of miRNAs and their corresponding targets make it possible to understand the gene regulation networks and miRNA-mediated biological mechanisms. This technology has been used in *Arabidopsis* and also agricultural crops like rice, alfalfa, and tomato for functional genetic studies (Warthmann et al. 2008; Haney and Long 2010; Zhang et al. 2011). But only a limited number of reports targeting members of a multigene family (Haney and Long 2010; Kim and Somers 2010) are available.

7.3 miRNAs and Potato Tuber Browning

Prior to the use of miRNA approach in studying the PPOs, several studies used small [or short] interfering RNA (siRNA) approach to knockdown PPO gene expression by transformation with hundreds of base pairs of the PPO gene fragments (Bachem et al. 1994; Coetzer et al. 2001; Murata et al. 2001; Thipyapong et al. 2004; Wahler et al. 2009; Richter et al. 2012a, b). The strategies were definitely successful in suppressing the overall PPO activities and reduction of PPO-mediated browning reactions, but this approach was unable to identify the role of the individual PPO gene members. The siRNAs generated in the transgenic plants were not targeted to a particular PPO gene (as opposed to targeted silencing in miRNAs) but affected other members of the PPO multigene family sharing high homology in nucleotide sequences (Mayer 2006; Tran et al. 2012).

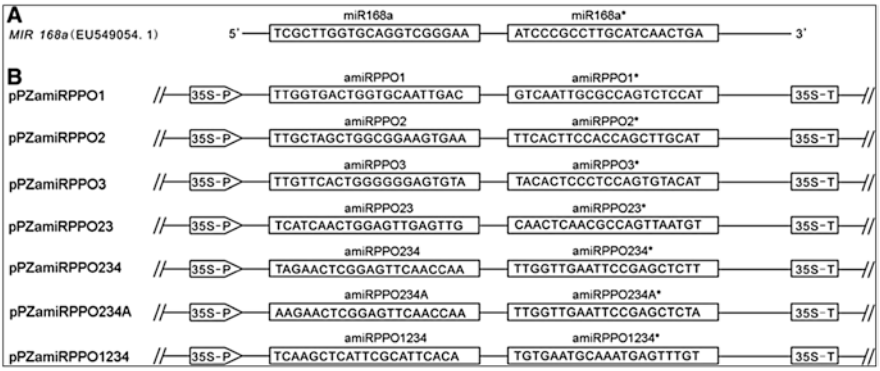

Fig. 7.5 Diagrammatic representation of artificial microRNA constructs. (**a**) Linear structure of the miR168a primary transcript gene (MIR168a, nt 120–355, GenBank Accession No. EU549054.1). Sequences of the miR168a and its complementary region (illustrated as approximately miR168a*) in the gene are displayed in the *boxes*. (**b**) Structure of the binary vectors for expression of amiRNAs. Construct names are indicated at the *left*. The sequences of the designed amiRNA and its complementary region (approximately amiRNA*) are displayed in the *boxes*. 35S-P, CaMV 35S promoter. 35S-T, CaMV 35S terminator (Source: Chi et al. 2014, BMC Plant Biology 14: 62)

The artificial miRNA (amiRNA) approach was utilized to suppress the potato (*Solanum tuberosum*) PPO multigene family consisting of *POTP1/POTP2, POTP32, POTP33,* and *POTP72* (Chi et al. 2014). The suppression was carried out individually and in combination of genes using amiRNAs to study the contribution of potato PPOs to total PPO activity and also to understand the correlation of protein/activity levels with browning of tuber tissue. As discussed earlier, Chi et al. (2014) identified nine PPO-like gene models, *StuPPO1 to StuPPO9*. Based on these nine PPO-like and earlier reported PPO genes, seven different amiRNAs were designed: *StuPPO1* (earlier named *POTP1/POTP2*), *StuPPO2* (previously *POT32*), *StuPPO3* (previously POT33), and *StuPPO4* (earlier *PPO72*) in potato (1, 12). The amiRNA sequences designed were complementary to either a particular PPO or multiple targets by choosing the suitable 21-bp region of the corresponding PPO. The seven amiRNAs constructs were developed by incorporating them in an *Arabidopsis thaliana* mi168a transctipt gene (Fig. 7.5). The transgenic lines developed were utilized for molecular screening and analysis. The corresponding amiRNAs were not detected in transgenic amiRPPO2 series lines (for amiRPPO2) and amiRPPO234 series lines (for amiRPPO234) for unknown reasons. Several transgenic potato lines exhibited a substantial downregulation of targeted PPO gene expression.

The real-time quantitative reverse transcription PCR (qRT-PCR) assay revealed that the transcript levels of the *StuPPO1* genes were decreased by 68–98% in six amiRPPO1 series transgenic lines (clones). The amiRPPO1 series was designed to express amiRNA-amiRPPO1 targeted to *StuPPO1*. Similarly, one amiRPPO2 (target: *StuPPO2*), two amiRPPO3 (*StuPPO3*), five amiRPPO23 (*StuPPO2* and *StuPPO3*), two amiRPPO234 (*StuPPO2*, *StuPPO3*, and *StuPPO4*), three

Table 7.1 Exceptions to targeted gene silencing using amiRNA for potato PPOs

S.No	amiRNA series – plant number	Target PPO gene	Reduction in nontarget PPO expression (%)
1	amiRPPO1-2	*StuPPO1*	*StuPPO2* by 50%; *StuPPO3* by 80%
2	amiRPPO3-12	*StuPPO3*	*StuPPO1* and *StuPPO2* by 50–60%
3	amiRPPO3-15	*StuPPO3*	*StuPPO4* by ~60 %
4	amiRPPO23-5,-7,-9	*StuPPO2* and *StuPPO3*	*StuPPO4* by 80%
5	amiRPPO234A-4,-6,-14	*StuPPO2* to *StuPPO4*	*StuPPO1* by 50%

Source: Chi et al. (2014), BMC Plant Biology 14: 62

amiRPPO234A (*StuPPO2, StuPPO3*, and *StuPPO4*), and five amiRPPO1234 (*StuPPO1, StuPPO2, StuPPO3*, and *StuPPO4*) series lines exhibited significant reduction of transcript levels of the target genes. The miRNA-based RNA silencing in plants was shown to take place through complementary cleavage of targeted mRNA by Argonaute proteins (Rhoades et al. 2006). Using 5′-RACE PCR, the cleavage of PPO gene transcript was identified to be a 253-bp product that had 97–99% nucleotide sequence identity with *StuPPO1* gene. Further, the cleavage site was between 10th and 11th nucleotide at the amiRPPO1 site. Based on these results, it can be concluded that amiRNAs functioned as the small RNAs that were responsible in silencing of the target gene of interest. The results demonstrated that potato PPO isoforms can be individually or simultaneously suppressed using amiRNAs approach. Interestingly, the amiRNAs downregulated some nontarget PPO genes in addition to their targeted genes (Table 7.1). Overall, the expression of targeted genes was reduced by 75–100% compared to the wild-type plants. The independent or collective downregulation of PPO genes did not generally cause upregulation of other PPOs, except for transgenic line amiPPO1-12 that showed a 90% decrease in the target gene, *StuPPO1*, and ~40% increase in the non-target *StuPPO2* gene transcript.

The total PPO protein level in transgenic potato tuber relative to that in the non-transgenic tubers, i.e., relative PPO protein level (RPR), similarly relative PPO activity (RPPO), and relative browning potential (RBR) were analyzed to understand the correlations among these characters. The percentage reduction of the three traits is presented in Table 7.2.

The browning phenotypes exhibited by air-exposed freshly cut potato tubers were consistent with the browning potential results obtained (Fig. 7.6). Among the different transgenic plant types, amiRPPO1 and amiRPPO3 exhibited stronger browning phenotype (based on the degree of dark color development) followed by amiRPPO23, amiRPPO234A, and amiRPPO1234.

Pearson's correlation coefficient (r^2) indicated that *StuPPO2* was highly correlated with RPR, RPPO, and RBR ($r^2 = 0.70–0.80$, $P < 0.0001$), followed by moderate association of *StuPPO3* and *StuPPO4* ($r^2 = 0.59–0.71$, $P < 0.0001$), while the *StuPPO1* gene had a weak correlation ($r^2 = 0.19–0.27$, $P < 0.05$). Despite the failure

Table 7.2 Reduction (%) in RPR, RPPO, and RBR in different transgenic potato lines

Transgenic lines	Reduction (%)		
	RPR	RPPO	RBR
amiRPPO1-2,-3-12	23–30	25–35	35
amiRPPO3-12,-15	15–20	15–25	10
amiRPPO23-5,-7,-12	45–70	75–95	50–65
amiRPPO234A-4,-6-14			
amiRPPO1234-2,-6,-12	73–80		65–75

Source: Chi et al. (2014), BMC Plant Biology 14: 62

to generate *StuPPO2*-knockdown mutants, it appears that *StuPPO2* protein likely contributes ≥55% to total PPO protein levels in non-transgenic lines and also 70% of the variability in total PPO protein level tuber that was explained by *StuPPO2*. The contributions of different potato genes toward the total PPO gene transcript levels in the non-transgenic tubers were *StuPPO2* (67%) > StuPPO1 (28%) > *StuPPO3* (4%) > StuPPO4 (<0.2). Gene transcript levels in the non-transgenic tubers were *StuPPO2* (67%) > StuPPO1 (28%) > *StuPPO3* (4%) > StuPPO4 (<0.2). Based on these results, *StuPPO2* is suggested to be the major contributor to the PPO protein content in the wild-type potato tubers. Finally, it can be concluded that the miRNA approach has very clearly demonstrated for the first time that the four potato PPO genes (*StuPPO1, StuPPO2, StuPPO3,* and *StuPPO4*) have to be suppressed simultaneously to realize optimal inhibition of PPO activity and non-browning phenotype in transgenic potato tuber tissue.

7.4 miRNAs in *Salvia miltiorrhiza*

Salvia miltiorrhiza Bunge is a familiar plant species used in various traditional Chinese medicines widely used to treat cardiovascular, hyperlipidemia, cerebrovascular, and acute ischemic stroke diseases (Li et al. 2009). It is also an emerging model system for medicinal plant biology (Song et al. 2013). The major active pharmaceutical ingredients in *Salvia miltiorrhiza* are hydrophilic phenolic acids; therefore understanding the regulatory mechanisms, metabolism, and biosynthesis of phenolic acids is important for quality improvement (Hou et al. 2013). Despite knowing the importance of PPOs in several plant species, no information is available for *Salvia miltiorrhiza.* Li et al. (2017) have identified 12 full-length and 7 partial *Salvia miltiorrhiza* PPO genes (SmPPO). The full-length sequences of all the 19 SmPPOs were obtained, and the identified SmPPOs were annotated as *SmPPO1* to *SmPPO19*.

Salvia miltiorrhiza with 19 PPO genes could be the largest multigene family of plant PPOs identified so far. The *SmPPO* expansion is mostly a consequence of lineage-specific gene duplication and subsequent divergence. Of the 19 genes identified, 17 SmPPOs cluster into six paralogous groups. Based on Ka and Ks values for open reading frames (ORF) and coding sequences for CuA/CuB domain, DWL domain, and KFDV domain, it is suggested that *SmPPOs* are highly conserved. The

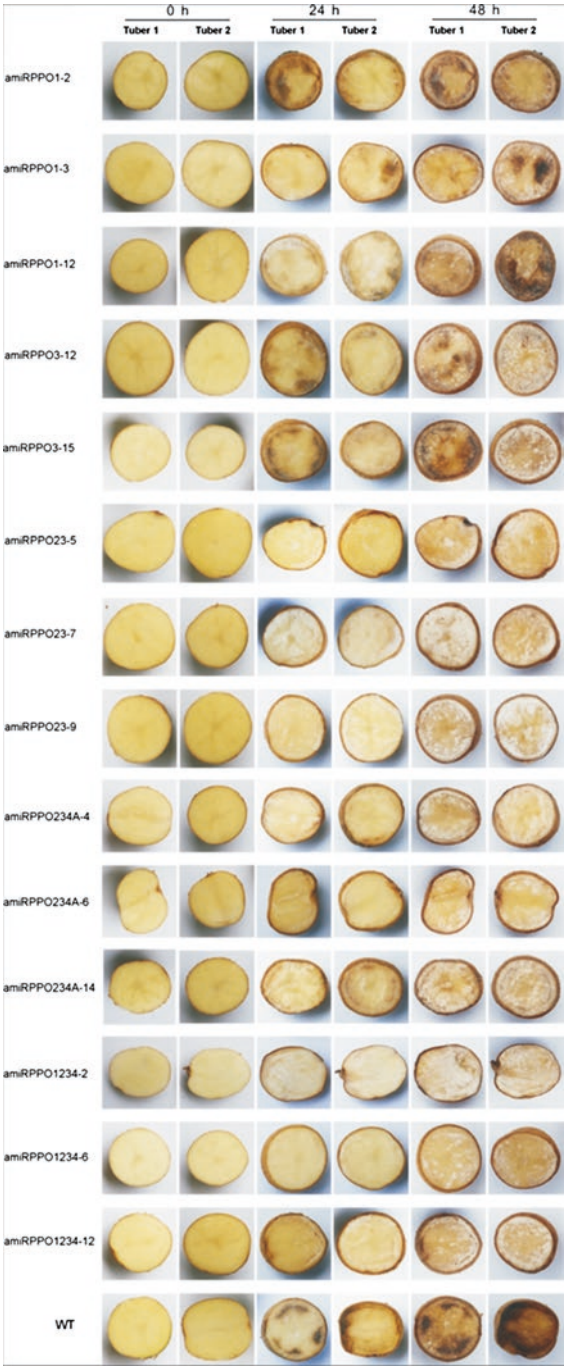

Fig. 7.6 Browning phenotypes. Potato tubers from each transgenic line and wild type were randomly selected, cut into approximately two equal sections, and exposed to air/oxygen at room temperature (~25 °C). Images were taken at 0-, 24-, and 48-h post air exposure. Browning or blackening typically started from vascular ring region and advanced to the medullary tissue with the air exposure time (Source: Chi et al. 2014, BMC Plant Biology 14: 62)

gene structure studies indicated the presence of introns (0–2) in the coding regions of *SmPPOs*. Four genes (*SmPPO8*, *SmPPO12*, *SmPPO16*, and *SmPPO17*) had an intron in the CuA domain-encoding region, whereas *SmPPO5* had an intron in the PPO_DWL domain-encoding region. *SmPPO15* had two introns, each located in the CuA and PPO_DWL domain-encoding regions. The levels of *SmPPO* transcripts were detected in different tissues like leaves, stems, flowers, and roots to identify their physiological roles. As expected differential expression of *SmPPOs* was observed. Several genes exhibited strong expression in leaves (*SmPPO3*, *SmPPO7*, *SmPPO8*, *SmPPO14*, and *SmPPO17*) and flowers (*SmPPO6*, *SmPPO9*, *SmPPO10*, *SmPPO12*, *SmPPO13*, and *SmPPO15*). While *SmPPO1* and *SmPPO16* were largely expressed in roots, *SmPPO11* was predominant in stems. The remaining five *SmPPOs* were mostly expressed in at least two tissues.

S. miltiorrhiza roots are widely used in traditional Chinese medicines. Lithospermic acid B is the pharmacologically active phenolic acid that accumulates mostly in the phloem and xylem of *S. miltiorrhiza* roots (Xu et al. 2015). The transcriptome-wide analysis (RNA-seq) of *S. miltiorrhiza* showed that eight *SmPPOs* exhibited higher expression levels in the phloem and xylem than the periderm probably suggesting their putative role in lithospermic acid B biosynthesis. The RPKM value of the eight *SmPPOs* was greater than 2 (Gao et al. 2014). The methyl jasmonate (MeJA) is an elicitor of tanshinone and phenolic acid production in *S. miltiorrhiza* and is involved in plant response to stress (Gao et al. 2014; Xiao et al. 2009). The *SmPPOs* showed differential response to MeJA treatment with 11 PPOs (*SmPPO1*, *SmPPO2*, *SmPPO3*, *SmPPO5*, *SmPPO8*, *SmPPO9*, *SmPPO10*, *SmPPO12*, *SmPPO14*, *SmPPO15*, and *SmPPO17*) being significantly upregulated, while five (*SmPPO4*, *SmPPO6*, *SmPPO13*, *SmPPO16*, and *SmPPO19*) were downregulated, and two PPOs (SmPPO11 and SmPPO18) exhibited fluctuation in their response. Additionally, 12 SmPPOs were responded to the yeast extract and Ag^{+} treatment, a combination of biotic and abiotic stress (Gao et al. 2014).

In *Salicaceae*, a lineage-specific young miRNA termed miR1444 regulates a subset of PPOs (Lu et al. 2008, 2011; Wang et al. 2017). The regulation of *P. trichocarpa* PPOs (*PtPPOs*) by MiR1444 is important in copper homeostasis and stress responses (Lu et al. 2011). Therefore to examine the role of miR1444 in *SmPPO* regulation, the high-throughput sequencing data (of leaves, flowers, stems, and roots) obtained from this work and already published data (Xu et al. 2014; Shao et al. 2015) were analyzed with no success. Additionally, no precursor sequence of miR1444 was identified from the whole genome sequence data of *S. miltiorrhiza* (Xu et al. 2016). These results indicate that there is no miR1444 in *S. miltiorrhiza*, and therefore the SmPPOs are not regulated by miR1444. Since miR1444 was not involved in SmPPO regulation, Li et al. (2017) searched for *S. miltiorrhiza* small RNAs possibly targeting *SmPPOs* for cleavage using psRNATarget (Dai et al. 2011). A total of 54 small RNAs were identified, aligned with the whole genome sequence of *S. miltiorrhiza*, and the secondary structures were predicted as described previously (Lu et al. 2005). The structures were annotated following the criteria described by Meyers et al. (2008) which helped in identifying a novel miRNA stem-loop structure designated as Smi-MIR12112 (Fig. 7.7).

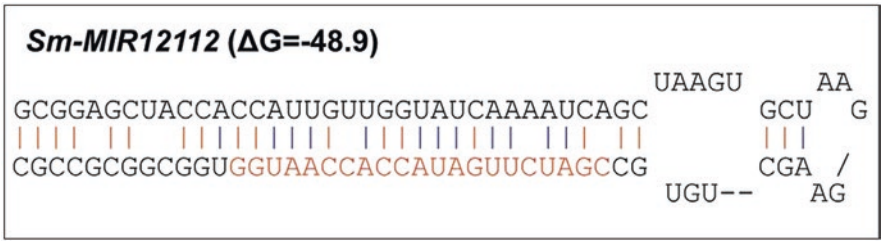

Fig. 7.7 Predicted hairpin structures of Smi-miR12112. Mature miRNA sequences are indicated in *red* (*Source*: Li et al. 2017, Scientific Reports 7: 44622)

Smi-MIR12112 showed higher expression levels in young tissues of leaves, stems, and roots compared to the matured tissues indicating its importance mostly in the young tissues. Interestingly, Smi-MIR12112 also showed a differential response to MeJA treatment as exhibited by *SmPPOs* suggesting the complex nature of gene regulatory networks associated with miRNA and *SmPPOs*. Further, in silico target prediction showed that 15 SmPPOs had an almost perfect complementary sequence to Smi-MIR12112 (Fig. 7.8). The complementary sequence was located in the region-encoding KFDV domain, a highly conserved domain among PPOs. But, the target sites for Pt-miR1444 and Vv-MiR058 were located in the regions encoding CuB and thylakoid transfer domain.

Usually, the target mRNAs are cleaved by the RNA-induced silencing complexes (RISCs) at the tenth complementary nucleotide from the 5′ end of the miRNA (Rhoades et al. 2002). The analysis of *S. miltiorrhiza* degradome sequencing data confirmed that the 15 *SmPPOs* are targets of Smi-miR12112. Further, the results from rapid amplification of 5′ complementary DNA ends (5′-RACE) showed that five Sm*PPOs* (*SmPPO3*, *SmPPO5*, *SmPPO9*, *SmPPO11*, and *SmPPO13*) were indeed cleaved by Smi-miR12112 in vivo, confirming the results obtained from computational prediction. Usually the miRNA target sites occur outside of family-defining/conserved domains (Allen et al. 2004). Therefore, the origin and evolution of miRNAs targeting conserved domains of PPO may be under strong negative selection. It indicates the importance of miRNA-mediated posttranscriptional regulation of PPOs in plants.

7.5 Mutagenesis Studies of Polyphenol Oxidases

Polyphenol oxidases (PPOs) are important class of type 3 copper enzymes containing two copper atoms (CuA and CuB) and bind oxygen molecules in a characteristic "side-on" bridging mode (μ-η^2: η^2) between both copper atoms (Kitajima et al. 1989). The PPOs are classified into tyrosinases [monophenol, o-diphenol: oxygen oxidoreductase, EC 1.14.18.1], catechol oxidases [CO, *o*-diphenol:oxygen oxidoreductase, EC 1.10.3.1], aureusidin synthase [2′,4,4′,6′-tetrahydroxychalcone 4′-O-β-$_D$-glucoside:oxygen oxidoreductase, EC 1.21.3.6], and laccases [benzenediol:oxygen

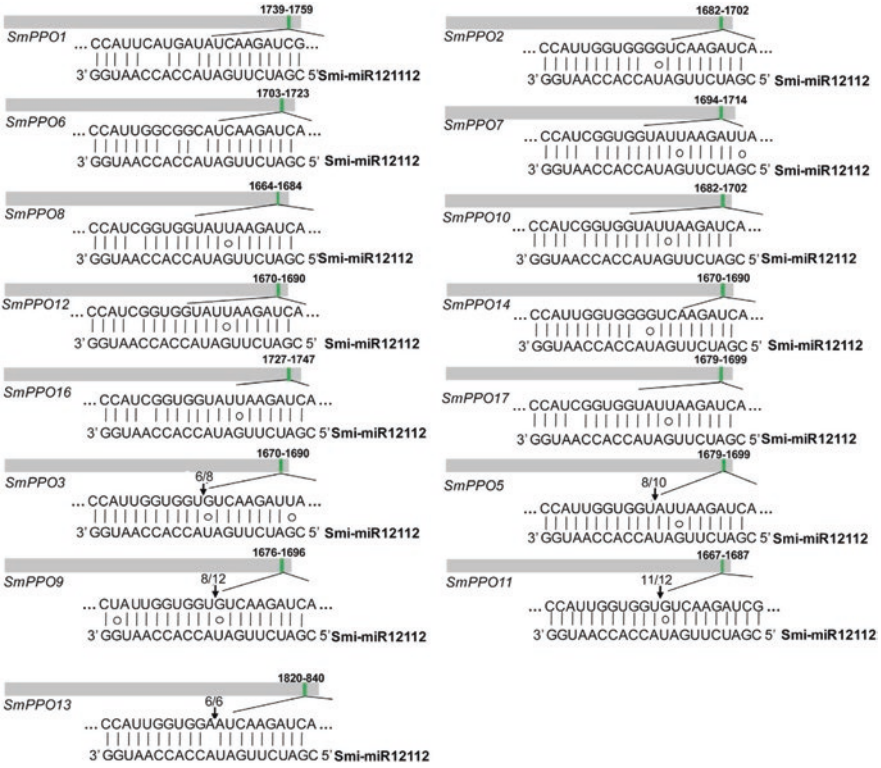

Fig. 7.8 Locations of Smi-miR12112 complementary sequences in *SmPPOs*. Heavy *gray lines* represent ORFs. Smi-miR12112 complementary sites (*green*) with the nucleotide positions of *SmPPO* are indicated. The mRNA sequence of each complementary site from 5′ to 3′ and the mautre Smi-miR12112 sequence from 3′ to 5′ are shown in the expanded regions. Watson-Crick pairing (*vertical dashes*) and G:U wobble pairing (*circles*) are indicated. *Vertical arrows* indicate the 5′ termini of miRNA-guided cleavage products, as identified by 5′-RACE, with the frequency of clones shown (*Source*: Li et al. 2017, Scientific Reports 7: 44622)

oxidoreductase, EC 1.10.3.2] (Mayer et al. 2006). Tyrosinases, catechol oxidases, and laccases have already been discussed in the earlier chapters. Aureusidin synthase (AmAS1) and aurone synthase are unique PPOs as they are involved in aurone synthesis (Kaintz et al. 2014; Nakayama et al. 2000). Aureusidin synthase was found in the 4-hydroxyaurone pathway of *Antirrhinum majus* and *Helichrysum bracteatum*; it is involved in the hydroxylation and the oxidative cyclization of 2′,3,4,4′,6′-pentahydroxychalcone (PHC) and 2′,4,4′,6′-tetrahydroxychalcone (THC) into aureusidin and bracteatin, respectively (Nakayama et al. 2000; Nakayama 2002; Ono et al. 2006). Aurone synthase found in *Coreopsis grandiflora* (cgAUS1) is involved in the 4-deoxyaurone pathway and catalyzes only the conversion of chalcones to aurones, by oxidizing butein (6′-deoxychalcone) to sulfuretin (4-deoxyaurone; Kaintz et al. 2014; Miosic et al. 2013). Thus, aurone synthase accepts only dihydroxylated substrates (hydroxyl groups at third and fourth position

of ring B), while aureusidin synthase accepts both monohydroxylated and dihydroxylated substrates (Kaintz et al. 2014; Miosic et al. 2013). Aurone synthase contains 602 amino acids (includes transit peptide) with a corresponding molecular mass of 68 kDa (Kaintz et al. 2014).

A very limited number of mutagenesis studies of recombinant PPOs are reported and specifically are rare in plant PPOs. The first site-directed mutagenesis study in plant PPOs was performed of dandelion (*Taraxacum officinale*; Dirks-Hofmeister et al. 2012). In this study Dirks-Hofmeister et al. (2012) identified a surface-exposed cysteine (C197; not present in cgAUS1) by analyzing a tetrameric PPO isoenzyme (PPO-6) through molecular modeling. A cysteine (C197) to a serine mutation proved that this amino acid residue stabilizes the tetrameric enzyme through a disulfide linkage (Dirks-Hofmeister et al. 2012). Though the serine mutant form of the enzyme formed a tetrameric structure, the enzymatic efficiency and cooperativity were impaired with a concurrent reduction in enzyme stability (Dirks-Hofmeister et al. 2012). Site-directed mutagenesis on all copper-coordinating histidines, the seventh, conserved non-coordinating histidine in CuB, and the thioether bridge-building cysteine of a fungal tyrosinase (*A. oryzae* TYR, *ao*TYR; Nakamura et al. 2000) with asparagine resulted in mutated enzymes with no activity and having only one copper ion per molecule tyrosinase. The results from fungal tyrosinase studies indicate the importance of the mutated residues in copper incorporation and activity. The studies on phenolase/diphenolase activity of *Bacillus megaterium* tyrosinase (*bm*TYR) suggested that the less bulky valine (V218; not present in catechol oxidases, aureusidin synthase, or aurone synthase) allows the hydroxylation of monophenols at CuA (Goldfeder et al. 2013). Interestingly, the site-directed mutagenesis of V218 with a phenylalanine (V218F; corresponds to F273 in cgAUS1) resulted in increased monophenolase activity (Goldfeder et al. 2013).

The three copper-coordinating histidines (H93, H116, and H125; Fig. 7.9) were mutated with alanine (H93A, H116A and H125A) to demonstrate their essential role in copper incorporation and folding (Kaintz et al. 2015). Mutation of the phenylalanine (F273; Fig. 7.9) to alanine (F273A) should elucidate if the phenylalanine's side chain functions as a blocker residue in cgAUS1 thereby influencing the monophenolase/diphenolase activity (Mauracher et al. 2014; Goldfeder et al. 2013; Fujieda et al. 2013). The cysteine (C97) which was suggested to form a thioether bridge with H116 (Kaintz et al. 2014; Klabunde et al. 1998) was mutated to alanine (C97A) to show its impact on structural restraints on the ligand sphere of the CuA center. Additionally, all the three coordinating histidines of CuB center were also mutated to alanine (H252A, H256A and H286A). As expected the copper content of the wild-type cgAUS1 was ~2 Cu per molecule cgAUS1. The three histidine mutants of CuA (H93A, H116A, H125A), F273A mutant, and C97A mutant contained only one Cu, two Cu, two Cu per molecule cgAUS1, respectively. The mutants of three coordinating histidines (H63, H84, and H93) and cysteine (C82) in CuA site of *ao*TYR exhibited a 50% decrease in copper binding indicating that these mutants had only one Cu per molecule of enzyme (Nakamura et al. 2000).

These results indicate that the mutation of cysteine residue is not essential for copper incorporation, but the thioether bond is important for enzyme expression,

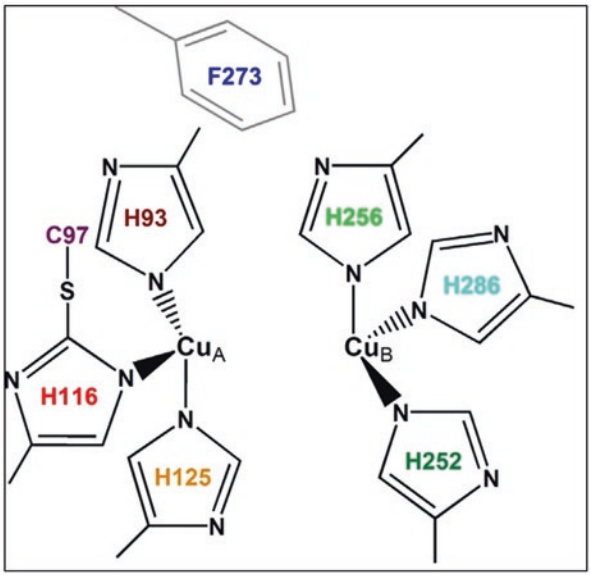

Fig. 7.9 Illustration of type 3 copper center of cgAUS1 (Source: Kaint et al. 2015, FEBS Letters 589: 789–797)

folding, and enzymatic analysis. The decrease in copper content to one Cu per molecule and loss of monophenolase/diphenolase activity suggest that the three copper-coordinating histidines are essential for copper binding and catalysis in *ao*TYR (Nakamura et al. 2000), and this is further confirmed in cgAUS1 (Kaintz et al. 2015).

The wild-type cgAUS1showed diphenolase activity as expected. But none of the mutants of cgAUS1 showed any diphenolase activity with any of the dihydroxylated substrates (butein, fisetin, or TBC) tested (Kaintz et al. 2014). As expected monophenolase activity was not observed in both the wild and mutant types. The bulky phenylalanine (F273) was assumed to be a blocking residue at the CuA site hindering monophenolic substrates in catechol oxidases (Klabunde et al. 1998; Matoba et al. 2006; Mauracher et al. 2014). This assumption was endorsed by the presence of a much smaller alanine residue in *Agaricus bisporus* tyrosinase (*ab*TYR) in place of phenylalanine in *Ipomoea batatas* catechol oxidase (*ib*CO; 16, 18, 49). But the F273A mutants of cgAUS1 lacked both monophenolase and diphenolase activity disapproving the role of phenylalanine as a blocking residue for monophenols probably indicating the role of F273 in substrate binding. The analysis of *bm*TYR mutant of valine with phenylalanine showed an increase in the enzyme activity contradicting the proposal of phenylalanine being the blocking residue for monophenolic substrates (Goldfeder et al. 2013; Fujieda et al. 2013). Overall, the site-directed mutagenesis of the all the amino acids (H93, H116, H125, F273, C97, H252, H256, H286) is essential for enzyme function and substrate binding. The F273 and C97 are probably essential either for substrate binding or for binding in the correct

orientation for the reaction. Kaintz et al. (2015) suggested twofold importance of C97 residue: (i) for correct protein folding and (ii) for diphenolase activity. Further, proposed F273 is important for substrate binding, but it is unable to induce tyrosinase-like reaction mechanism.

A vast amount of information regarding the identification and characterization of plant PPOs is already available from various sources. But during the last decade or so, significant new and novel information regarding the genetics, genomics, structure, functions, and modifications of PPOs in general and plant PPOs in particular has immensely helped us to better understand their biological roles. Despite these substantial achievements, there are a few more lacunae like more detailed structural information that would aid in better understanding their physiological functions.

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