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# RESEARCH ON CATECHOLASES, LACCASES AND CRESOLASES IN PLANTS. RECENT PROGRESS AND FUTURE NEEDS

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Polyphenol oxidases (PPOs) reveal a range of forms and occur in all plants and crops. PPOs are comprised of three enzymes (catecholase, laccase, cresolase) with very different activities and specificities. Cresolase has a dualistic form (cresolase is only in plants and tyrosinase is only in animals and microorganisms). Very often in the literature the generic word "PPO" is used inappropriately as one enzyme. This should be avoided in future studies, as clear systematics and correct nomenclature of PPOs are needed for proper research. PPOs have different substrate specificities and typical inhibitors, and they catalyze hydroxylation and oxidation processes in plants. Pigment formation in cells and cellular systems is affected by active PPOs. Catecholases, laccases and cresolases are encoded by nuclear genes of plants. Various PPO DNA sequences have been found, and PPOs occur in multiple gene families. The protective potential of PPOs in plants and enhanced herbivory resistance is debated, and the final evidence has not yet appeared. The activity of PPOs in germination is recognized, but its mechanism is still not clear. Seed testa coloration in *Arabidopsis thaliana* is effected by laccase and not by catecholase. The TT10 gene encoding laccase in the *Arabidopsis* seed testa has been isolated. *Arabidopsis* genome analysis led to the identification of 16 other putative laccases and their genes, named *AtLAC1* to *AtLAC17* according to their position in the genome. Challenging areas of research for the future are seed testa PPOs and their mobilization in endosperm and micropylar regions, and PPOs as a part of the plant defense system and immunity.

**Key words:** Plant polyphenol oxidase, catecholase, laccase, cresolase, coloration, plant enzyme.

## INTRODUCTION

Catecholase (EC 1.10.3.2), laccase (EC 1.10.3.1), cresolase (EC 1.14.18.1 in plants) and tyrosinase (EC 1.14.18.1 in animals) are polyphenol oxidases (PPOs). Cresolase and tyrosinase are the same enzyme differentiated according to their organism specialization: cresolase occurs in plants but not in animals, and tyrosinase occurs in animals but not in plants. Catecholase and laccase differ from each other and from cresolase and tyrosinase. Although there is still not clear evidence at present to indicate that these proteins are fundamentally different in structure or that there are separate genes coding for these enzymes, there is clear evidence that these enzymes have different activities. Very often in the literature the generic word "PPO" is used inappropriately as one enzyme. This should be avoided in future studies, as clear systematics and correct nomenclature of PPOs are needed for proper research. PPOs are comprised of three different enzymes, of which one is dualistic in form (cresolase, tyrosinase). These three different enzymes are widely distributed in nature and are important enzymes in plants, fungi, bacteria and animals, although their biological functions and mechanisms are not known in detail (Mayer, 2006). Recently the genes encoding these enzymes were found in bryophytes (Richter et al., 2005), but the mechanism of their activity, especially PPO activity in *Physcomitrella patens*, is not known and continues to be the object of intensive investigation. One prob-

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lem is that the PPO substrate has not yet been found in this plant, and the problem of gene expression is still open to discussion. PPOs have generally been recognized in many studies as important enzymes in all organisms, especially in plant life, seed and fruit formation (Vaughn and Duke, 1984; Mayer, 1987; Vaughn et al., 1988; Lee et al., 1990; Dijkstra and Walker, 1991; Martinez and Whitaker, 1995; Sherman et al., 1995; Withaker and Lee, 1995; Marshall et al., 2000; Strack and Schliemann, 2001; Yoruk and Marshall, 2003; Gandía-Herrero et al., 2005a; Jukanti, 2005; Jukanti et al., 2006; Berbehenn et al., 2007; Chang et al., 2007). From the time of Yoshida (1883), who discovered these enzymes in his research on the chemistry of the lacquer tree, PPOs have been important enzymes not only in the sciences, botany and chemistry, but also in enzyme technology and plant production, food trade and food consumption, as they are known to influence the external and internal quality of fruits and vegetables, involving real and potential economic benefits and losses (Marshall et al., 2000). Moreover, this group of enzymes, especially laccases, is important as they also occur in animals, plants, bacteria and fungi, and can participate actively in establishing the pathogenicity process in plants and even directly in the degradation of lignin (Higuchi, 2004). Biochemical and biotechnological approaches to lignin biodegradation require knowledge of laccase molecular biology for possible bioengineering. Some authors include PPOs among the most studied enzymes (Mayer, 1987; Mayer and Staples, 2002; Yoruk and Marshal, 2003; Lei et al., 2004; Mayer, 2006). Thousands of reports on PPOs have been published since Yoshida (1883). Although clear progress in research on PPOs has been made in this period, there is still much to learn about the mechanisms of these enzymes in plants. In his review, Mayer (2006) has even stated that the biological functions of PPOs are still enigmatic. The methodological difficulties in research on PPOs, and especially plant PPOs, are exacerbated by many environmental factors possibly influencing the expression of this enzyme in different leaves, according to the cultivar, its age and growth, day length, humidity conditions and growing season (Shafran et al., 2007). The suggested differentiation of PPO activity according to growing conditions and environmental factors means that the research conditions for PPO research need to be standardized so that the results can be compared. An enormous number of reports have been published on PPOs to the present day, connected more with applied research than basic research in enzymology and biochemistry. The results published are not always comparable as the research conditions have differed. The research needs are still great, since these enzymes are not only scientific objects but also bioactive agents which reduce the commercial profits of basic fruits, berries and vegetables. This paper emphasizes recent progress in research on plant PPOs (catecholases, laccases, cresolases) and comments on areas still not sufficiently studied but important for science and for practical applications. Our present research interest in PPOs is connected with the potential role of PPOs in plant seed biology. A project on this subject is under way in the Research and Teaching Laboratory of the Faculty of Biosciences of the University of Joensuu. We analyzed research in this area in order to present the newest findings and express some new ideas on this fascinating but difficult research topic, which has a history of 125 years. In this review we present three different enzymes representing PPOs, and discuss their importance for current and future research in botany.

## THE NATURE OF PLANT PPOs

Catecholases (synonyms: *o*-diphenol oxidases, catechol oxidase, *o*-diphenol oxygen oxireductase, EC 1.10.3.2), laccases (*p*-diphenol oxidases, *p*-diphenol dioxygen oxireductase, EC 1.10.3.1) and cresolases (monophenol monooxygenase, EC 1.14.18.1) are enzymes of secondary metabolism and act in the metabolic processes of phenolic compounds derived from the shikimate pathway. PPOs (catecholases, laccases and cresolases) are enzymes with a dinuclear copper center (CuA and CuB) with copper ions and conserved N and C termini. Structurally, plant PPOs differ slightly from animal PPOs. According to Steffens et al. (1994), one of the differences between plant and animal PPOs is the level of conservation of CuA and CuB sites. Specific to plant PPOs is that their CuA site is more highly conserved (90% amino acid identity) than the CuB site (70% amino acid identity). Unlike animal PPOs, plant PPOs have a third His cluster, which is CuC. The CuC cluster is similar to CuA and CuB binding sites, although its functional significance is still open to discussion. Catecholases (EC 1.10.3.2) oxidize *o*-diphenols to *o*-diquinones (Fig. 1), laccases (EC 1.10.3.1) oxidize *o*- and *p*-diphenols (and other substrates) to *p*-diquinones (and other quinones, and semiquinones) (Figs. 2, 3), and cresolases (EC 1.14.18.1) oxidize monophenols to quinones (Chrisari et al., 2008) and catalyze hydrogen abstraction (Fig. 4). Each PPO differs in its substrate specificity and possibly its physiological role. This substrate specificity is connected to the kind of enzyme, the plant species, and the chemistry of the substrate (Lee et al., 1990; Jukanti, 2005; Schmitz et al., 2008). Moreover, different PPOs have different substrate specificities and typical inhibitors. Catechol, 4-methylcatechol, 4-tert-butylcatechol, L-DOPA (L-3, 4-dihydroxyphenylalanine), (-)-epicatechin, (+)-catechin, chlorogenic acid

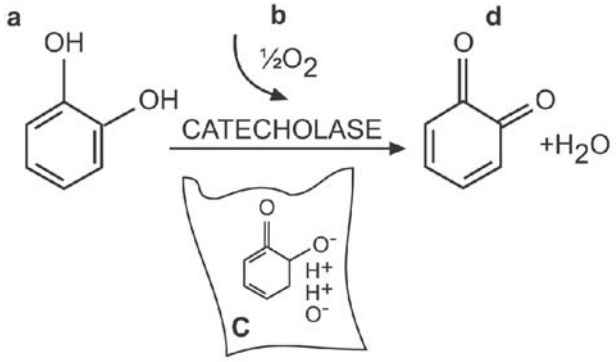


**Fig. 1**. Reaction catalyzed by catecholase (o-diphenol oxidase) with stages a-d. Explanations: Stage **a** – *o*-diphenol molecule with two hydroxyl groups. Stage **b** – activity of catecholase. One atom of oxygen is inserted into the hydroxyl groups and catecholase is active. Stage **c** – hydrogen atom abstraction and oxygen bond assimilation by ring. The second hydroxyl group is weakened and destroyed by the second hydrogen atom abstraction and oxygen bond assimilation. Stage **d** – oxidation is finalized by the strengthening of oxygen bonds with the ring and the reorganization of bonds in the ring. Quinone and water are finally produced.

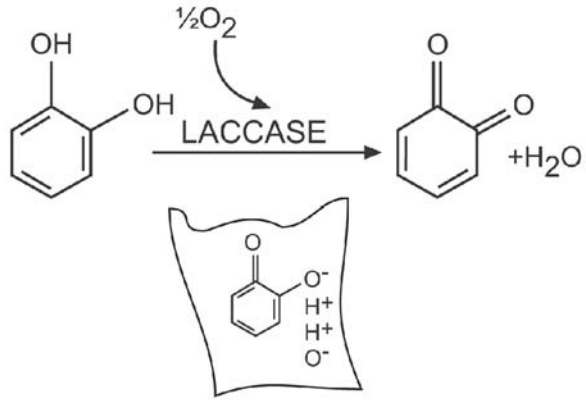


**Fig. 2**. The reaction catalyzed by laccase (*p*-diphenol oxidase). Explanation: Laccases oxidize *o*-diphenols to quinones. The reaction stages are the same as in the case of catecholase. Moreover, laccases can also oxidize a very wide range of substrates including triphenols and ascorbic acid (not shown). Laccases do not oxidize tyrosine and *p*-cresol (not shown).

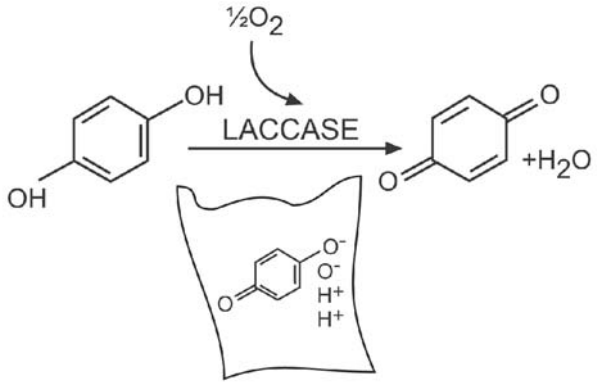


**Fig. 3**. The reaction catalyzed by laccase (*p*-diphenol oxidase). Explanation: Laccases oxidize *p*-diphenols, and *m*-diphenols to semiquinones and quinones. The reaction stages are the same as in the case of catecholase. Moreover, laccases can also oxidize a very wide range of substrates including triphenols and ascorbic acid (not shown). Laccases do not oxidize tyrosine and *p*-cresol (not shown).

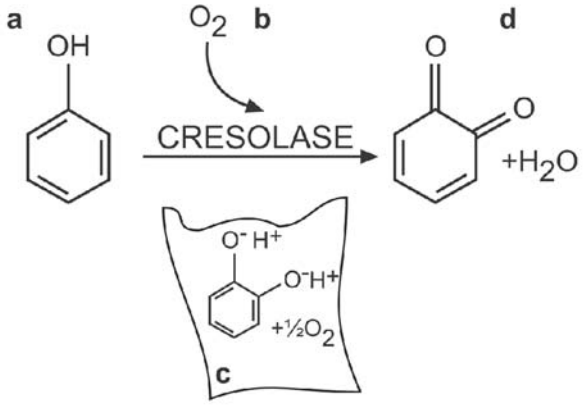


**Fig. 4**. Reaction catalyzed by cresolase (monophenol monooxygenase) with stages a–d. Explanations: Stage **a** – monophenyl molecule with hydroxyl group in the *ortho* position. Stage **b** – activity of cresolase. Two oxygen atoms are inserted into *ortho* position; hydroxyl group of monophenyl and the cresolase is active. Stage **c** – the first stage of catalyzing by cresolase. *Ortho* hydroxyl group is weakened and one atom of oxygen is inserted into the monophenol to start a possible second hydroxyl group. Both free hydrogen atoms are assimilated by one atom of oxygen. Stage **d** – oxidation is finalized by the assimilation of a second bond of oxygen atoms to the ring, where bond reorganization and strengthening is observed. Quinone and water are finally produced.

and dopamine are largely tested substrates for PPOs. However, the presence in vivo of several commonly tested substrates has still not been demonstrated, and the relevance of their physiological role remains uncertain. PPOs from red clover (*Trifolium pratense* L.) oxidize the physiological compound caffeic acid, an intermediate in the phenylpropanoid pathway and a central component of many *o*-diphenol esters or methoxy compounds (Schmitz et al., 2008). For catecholases, the most common physiological substrate in many plants, for example in *Allium* spp., *Morus alba* L., *Mentha piperita* L. and *Vitis vinifera* L., is catechol and its derivatives (Arslan et al., 1997; Kavrayan and Aydemir, 2001;

Unal and Sener, 2006; Rapeanu et al., 2006). It is catechin in, for example, *Camellia sinensis* L. (Halder et al., 1998), and 4-methylcatechol in *Lactuca sativa* var. *capitata* L. (Gawlik-Dziki et al., 2008). The mechanism of enzyme activity in plants is based on the interaction of the enzyme copper ions with molecular oxygen and the substrates.

Catecholases occur largely in plants but also exist in other organisms. Laccases are in certain higher plants although they are very typical for phytopathogenic fungi, and there are some exceptions in fruits and vegetables (Dijkstra and Walker, 1991). Cresolases only exist in plants but their activity is far less important than their counterparts in animals (including humans), which are tyrosinases (Marshall et al., 2000), the enzymes responsible for skin and hair coloration (eumelanins, phaeomelanins) (Strack and Schliemann, 2001). Cresolases and tyrosinases are the same enzymes but their activity and organism specificity differ. Moreover, the function of creating natural skin and hair pigments, melanins in the animal kingdom, also occurs in laccase (Suparno et al., 2007). Laccases are known as the enzymes responsible for polymerization of monolignols (Mayer and Staples, 2002), iron metabolism (Hoopes and Dean, 2004) and kernel browning processes in plants (Hood et al., 2003). Many of these functions, although mentioned in the literature, await more evidence.

All PPOs can be active or latent depending on activity conditions, substrates and inhibitors. Embryogenic cells are characterized by high initial PPO activity and the strong activation potential of the membrane-bound enzyme; non-embryogenic material reveals low phenolase activity and low activation potential (Grotkass et al., 1995). The latest studies suggest that yellow laccase is created by modification of blue laccase in the process of lignin degradation (Leontievsky et al., 1997). Although yellow laccase is artefactual, a modification of blue laccase, and does not exist as such in a normal organism, the potential diversity of laccases is a challenging point. Moreover, the molecular diversity of catecholases, laccases and cresolases in plants will be a very important future direction of research, as the nature of different laccases and possibly different catecholases and cresolases is still not known in detail, in spite of their wide distribution in the plant kingdom. PPOs probably occur in all plants and crops, with a range of multiple forms. For example, the range is reported to be three for alfalfa and red clover (Schmitz et al., 2008), three for hybrid poplar (Wang and Constabel, 2004), four for peach (Wong et al., 1971) and eleven for potato (Constantinides and Bedford, 1967; Wong et al., 1971). The ranges are species-dependent (Jukanti, 2005). Laccases in plants are involved in regeneration of tobacco protoplasts and in lignification of cell walls. Commercially, laccases have been used to delignify woody tissues, produce ethanol, and distinguish between such alkaloids as morphine and codeine, and can potentially be used in bioremediation processes in order to protect the environment from damage caused by industrial effluents (Mayer and Staples, 2002). Future results will open new possibilities to manipulate and produce new enzymatic molecules that not do exist naturally in plants.

All PPOs are encoded by nuclear genes of plants, and nearly all PPOs are transported to the thylakoid lumen of chloroplasts (Mayer and Harel, 1979; Steffens et al., 1994; Sommer et al., 1994; Sherman et al., 1995; Schmitz et al., 2008). The chloroplastic location of PPOs is presently widely accepted (Mayer, 1987, 2006; Schmitz et al., 2008). Note that the role of all three enzymes in chloroplasts has still not been established. Some studies do not pay enough attention to the differences in the functions of these three PPOs. Cresolase is sometimes omitted in PPO studies as it is mistakenly considered to be identical to catecholase. There are huge differences in the rates of activity and species specificity of, for example, cresolase hydroxylate and catecholase oxidate. Cresolase is equivalent to tyrosinase (animal and microorganism enzyme) and not to catecholase.

Catecholases, laccases and cresolases in plants can exist with low or undetectable activity, and should therefore be enhanced by fatty acids, mild heat, acid or alkali, proteolytic enzymes such as trypsin, detergents such as sodium dodecyl sulfate (SDS) or extended incubation (Schmitz et al., 2008). The protein size of PPOs depends on plant species and varies from 39 kDa in snapdragon to 73 kDa in spinach (Thipyapong et al., 2007). There are some differences between the size of latent and activated PPOs, but they are no larger than ~15 kDa at the C-terminal peptide extension (Robinson and Dry, 1992; Rathjen and Robinson, 1992). In comparison, the molecular weight of plant PPOs generally varies widely from 40 to 72 kDa (Steffens et al., 1994). Activation of these enzymes is based on the hypothesis that the properties of activation in vitro are comparable to those of activation in vivo. This hypothesis is strongly supported by comparison of *Beta vulgaris* PPO activation by trypsin digestion and by treatment with SDS (Gandía-Herrero et al., 2005b). PPO activity in vivo in plants is especially important in the case of crops and other useful plants, as their activity in growing plants is dependent on the growing conditions and cultivation practices. For example, in organically grown grapes it was double the level in conventionally produced grapes (Nunez-Delicado et al., 2005). This can be considered evidence (and the first scientific indicator) of a clear difference between products created by conventional and organic methods of production.

Future studies will clarify and determine the molecular diversity of PPOs and their links to the genotypic, metabolic and signalling machinery of plants. The molecular system of diverse PPO activation in relation to substrates and reaction energy and self-regulation connected with gene links remains to be clarified. Future studies on PPO inhibitors should also clarify the molecular mechanism of inhibition in relation to plant physiological inhibitors and the metabolic strategy of plants.

## HYDROXYLATION, OXIDATION AND COLORING PROCESSES IN PLANTS

PPOs are catalysts of hydroxylation and oxidation processes in plants (Marshall et al., 2000). Cresolases hydroxylate monophenols to *o*-diphenols and oxidize them to semiquinones. Hydroxylation in plants, especially in the applied sense, is a reaction considered less important than oxidation. The activity of cresolases is dependent on the substrate concentration, the pH and the presence of the existing catalytic amount of *o*-diphenols (Renes-Pinero et al., 2005). A pH of 5.0 is optimum for cresolases and the same has been reported for other PPOs in avocado (Espín et al., 1997). The activity of all PPOs is confirmed in this plant. Thus, hydroxylation and oxidation reactions probably are coupled in the course of ripening metabolism. In this sense, hydroxylation by cresolases seems to be very important for the activity of other PPOs (catecholases and laccases), although there is still no strong empirical evidence of this in other plant species. The kinetic behavior of cresolases in the plant kingdom is therefore a promising area of research, though not a simple one. The potential connection between cresolase activity and that of other PPOs suggests that it is part of the plant life strategy, but again this requires more research. The problems with in vivo substrates seem to be connected to the lack of deep cresolase studies, although Arslan et al. (2004) reported that in mulberry (*Morus alba* L.) no cresolase activity has been observed. In mushrooms, tyrosinase and catecholase activity in the same metabolic process has been shown, and inhibitors of their activities have been established (Saboury et al., 2006). The possibility that hydroxylation reactions by cresolases are only the first step in oxidation processes cannot be ruled out, as important as they are in both ripening and lignification of cells in plants. It is also clear, especially in mushrooms, that tyrosinases are able to metabolize aromatic amines and *o*-aminophenols (Toussaint and Lerch, 1987; van Gelder et al., 1997; Marshall et al., 2000). Ripening, darkening, browning and lignification of cells in plants have been shown to be affected by oxidation reactions resulting from the activity of catecholases and laccases (Marshall et al., 2000). Secondary polymerization reactions of quinonic compounds and proteins generate colorization in plants. This colorization of cells and cellular systems in organs is one of the most important life processes in plants. Color in plants can be affected in two ways: (1) by naturally occurring pigments (e.g., chlorophylls, carotenoids, anthocyanins); and (2) by pigments resulting from both enzymatic and non-enzymatic reactions (Marshall et al. 2000). Coloring reactions start only when the PPO occurs in the cytoplasm of the cells. Generally, coloring reactions cannot occur when PPOs are in the latent stage in plastids. Coloring reactions are connected to oxidation of phenols to *o*-quinones and their subsequent polymerization. High molecular weight compounds such as melanins (pigments) are a result of these oxidation and polymerization processes. Pigments can then react with amino acids and proteins, with the visible reaction of changing color. The components taking part in the reactions are oxygen, the PPO in its active form, copper and substrate. We know that PPOs can participate in plant pigment biosynthesis in two different pathways: betalain and aurone (Steiner et al., 1999; Strack and Schlieman, 2001). PPOs are involved in betalain biosynthesis in common portulaca (*Portulaca grandifolia* L.) and red beet (*Beta vulgaris* L.). Betalains are water-soluble pigments and aurones are gold-colored glycosides (pigments) occurring in the petals of some members of Astereceae, Scrophulariaceae, Cyperaceae and Fabaceae. These pigments take the place of anthocyanins, a class of flavonoids, which occur in all families of flowering plants. The mentioned pigments also include red-violet betacyanins and yellow betaxanthins. PPOs have been shown to play an active part in betacyanin synthesis occurring in the Chinese plant *Suaeda salsa* L. (Chenopodiaceae) seedlings growing in dark (Chang-Quan et al., 2007). Flavonoids, which are *ortho*-diphenols (*o*-diphenols), are substrates which can be oxidized to their corresponding semiquinones and quinones by PPOs. Semiquinones and quinones can react with phenols, amino acids or proteins, yielding a complex mixture of brown products (Walker and Ferrar, 1998; Pourcel et al., 2007). Moreover, non-enzymatic oxidation of flavonoids by auto-oxidation or chemical oxidation can also lead to the formation of quinoidal compounds; and through coupled oxidation reactions, quinones can oxidize other polyphenols which cannot be directly oxidized by enzymes. The formation of secondary quinones contributes to the formation of the heterogeneous polymers responsible for browning reactions (Guyot et al., 1996). Polyphenol oxidase (as a homologue of aureusidin synthase) can specifically catalyze the oxidative formation of aurones from chalcones, which are plant flavonoids responsible for the yellow

coloration of snapdragon (*Antirrhinum majus*) flowers (Nakayama et al., 2001; Ono et al., 2006). Moreover, PPOs are also known as rapid degraders of red pigments in lychee, a tropical fruit of high commercial value (Lattanzio, 2003). To degrade a red pigment or for the browning process to occur, enzymes have to be present in the same tissues and cell compartments with substrates. As in heated, non-senescent cells, where the enzymes are distributed in different subcellular compartments, the oxidation reaction only occurs after senescence or an environmental stress that destroys the biological barriers between the enzymes and the substrates (Yoruk and Marshall, 2003).

The processes of hydroxylation, oxidation and coloring in plants are known to be amenable to alteration by genetic engineering. This has practical significance in plant production and crop protection. By the means of genetic engineering it is possible to avoid tuber discolorations in the dominant potato (*Solanum tuberosum* L.) variety used for producing French fries (Rommens et al., 2006). These discolorations were linked to impact-induced bruising and were caused by the liberation of soluble and active PPOs from damaged plastids into the cytoplasm. A multigene silencing construct enhanced the performance of the studied potato variety in seven different ways: black spot bruise resistance, reduced cold-induced sweetening, reduced stress-induced sugar ends, enhanced fry aroma, reduced amounts of processing-induced acrylamide, reduced starch phosphate content, and increased starch. The sensory and nutritional characteristics of the potato were improved by simultaneously silencing the tuber-expressed polyphenol oxidase (*Ppo*), starch-associated (*R1*) and phosphorylase-L (*PhL*) genes. However, it is still not clear whether the color changes occurring by silencing PPO gene expression are genetically permanent over generations or if there are more phenotypic connections that will become less able to function in the next generations of plants.

The molecular mechanism of pigmentation reactions with diverse PPOs in different plant stages should be clarified in future studies. This mechanism is not clear in relation to phenological development and plant ageing strategy. The anticipated empirical data in this field can open new possibilities and horizons in theoretical and applied research. For example, it is known that seed testa coloration in *Arabidopsis thaliana* is affected by laccase and not by catecholase (Pourcel et al., 2005). Moreover, it is known that *TT10* laccase occurs in the *Arabidopsis* plant in young and colorless seed testa. In the ripening period of seeds, especially in the phase of desiccation and epicatechin oxidation, the quinones and their insoluble polymers lead to seed testa browning and water-impermeability (Pourcel et al., 2007). Enzymatic oxidation of polyphenols occurs during storage when cell integrity is affected. The gene *TT10* is involved in oxidative polymerization in *Arabidopsis*. Phenolic polymerization is a common function for plant laccases (Rouet-Mayer et al., 1990; Sterjiades et al., 1992; Wang et al.,2004; Pourcel et al., 2005). It is not known whether hydroxylation of menophenols to *o*-diphenols occurs in *Arabidopsis*.

## EXPRESSION OF GENES CODING PPOS

PPOs usually occur in gene families in higher plant species. The genes encoding PPOs have been isolated and investigated largely in a number of useful plant species such as wheat (*Triticum aestivum* L.) (Kruger, 1976; Demeke and Morris, 2002; Jukanti, 2005), poplar (*Populus* spp.) (Constabel et al., 2000), apricot (*Prunus* spp.) (Chevalier et al., 1999), tomato (*Lycopersicon esculentum* L.) (Thipyapong et al., 1997; Shahar et al., 1992), sugar cane (*Saccharum officinarum* L.) (Bucheli et al., 1996), potato (*Solanum tuberosum* L.) (Thygesen et al., 1995), grape (*Vitis vinifera* L) (Dry and Robinson, 1994), horse bean (*Vicia faba* L.) (Cary et al., 1992), moss (*Physcomitrella patens*) (Richter et al., 2005), banana (Gooding et al., 2001), apple (*Malus domesticus* Borkh.) (Boss et al., 1994; Kim et al., 2001), sweet potato (*Ipomoea batatas* L. (Lam) (Liao et al., 2006), red clover (*Trifolium pratense*), alfalfa (*Medicago sativa* L.) (Sullivan et al., 2004; Schmitz et al., 2008) and pear (*Pyrus pyrifolia* L.) (Nishimura et al., 2003). In many plants, such as wheat (*Triticum aestivum* L.), PPO activity is not controlled by a single gene but by multiple genes expressed in different tissues (Jukanti, 2005).

A multiplicity of genes and their differential expression in different plant parts and at different stages of development have been reported in many studies. Six different PPO DNA sequences falling into two clusters, each with three similar sequences (clusters of three genes), which are expressed in wheat kernels and regulate the darkening reaction in this species, were identified (Jukanti, 2005; Jukanti et al., 2004). Seven genes in different tissues of tomato were reported (Steffens et al., 1994; Thipyapong et al., 1997), and six genes coding the PPO of potato were also found (Thygesen et al., 1995). Two different genes expressed at different stages of apple flower development are also known (Kim et al., 2001). In older work, only the presence of a single PPO gene in grape vine was described (Dry and Robinson, 1994). A new cDNA encoding PPO (*IbPPO*) from sweet potato has also been found. This intron-free gene has a full-length cDNA of 1984 bp with 1767 bp encoding a 588-amino-acid

polypeptide with a calculated molecular weight of 65.7 kDa (Liao et al., 2006). *IbPPO* can be expressed in all organs of sweet potato, including mature leaves, young leaves, the stems of mature leaves (petioles), storage roots and veins, but at different levels. The highest level expression of *IbPPO* was in veins, and the lowest level in petioles; storage roots, young leaves and mature leaves showed moderate expression (Liao et al., 2006). The cDNA encoding three red clover PPOs were expressed in alfalfa (*Medicago sativa* L.) (Schmitz et al., 2008). The relationship between the variation of PPO genes and the PPO activity of seeds was investigated in wheat (*Triticum aestivum* L.) by Chang et al., (2007). The three genes expressed in immature wheat grain were investigated in 216 common wheat cultivars. The high polymorphism related to PPO activity was exhibited only by *TaPPO-A1* and *TaPPO-D1*. Two introns in these genes were found. During seed development, the high-PPO-activity cultivar showed higher transcription of two PPO genes than the low-activity cultivar. Variation in the intron may influence transcription of TaPPO-A1 and TaPPO-D1 in immature seeds of weeds (Chang et al., 2007).

PPO genes found in hybrid poplar (*Populus trichocarpa* × *Populus deltoids*) are expressed differentially under normal growing conditions. PtdPPO1 mRNA was absent without mechanical or chemical induction, whereas PtdPPO2 mRNA was found to be expressed in mid-veins, petioles, stems and roots. PtdPPO3 mRNA was expressed only in roots. PPO genes therefore have specialized physiological functions (Wang and Constabel, 2004). The mechanism of these specialized functions is not known. This seems to be more a hypothesis then scientific evidence.

The *TT10* gene was isolated in *Arabidopsis* seed testa. This gene encodes a putative laccase protein with 565 amino acids and a predicted molecular mass of 64 kD (Pourcel et al., 2005). *Arabidopsis* genome analysis led to the identification of 16 other putative laccases and their genes, which are named *AtLAC1* to *AtLAC17* according to their position in the genome. By analysis of laccase sequences, six different gene groups were established (McCaig et al, 2005).

Plants can be divided according to high and low PPO activity, and such variability is possible even within species. Different wheat (*Triticum aestivum* L.) cultivars had PPO molecular weights from 56 to 85 kD, and each of the genomes A, B and D contributed 2 PPO genes (Jukanti, 2005). A multigene family of PPOs is connected to the complex of the function of these genes and their transcriptional and post-transcriptional regulation. This seems to be a very important research area, and can help us to understand plant PPO functions and possible physiological specializations and also exceptions to these specializations at different developmental stages of plant growth. The results of Morris (2006) suggest that complete elimination of PPO activity from seeds would not completely eliminate wheat product discoloration. Moreover, genetic engineering provides a practical possibility of silencing the POT32 PPO gene by pSIM371 and pSIM217 gene manipulation and plant defect reparation (Rommens et al., 2006). This is a fascinating research direction.

## PROTECTIVE AND IMMUNITY POTENTIAL

Mayer (2006) concluded that the function of PPOs in plants remains unclear and in many cases is still not supported by published data, although a positive correlation between PPOs levels and resistance to pathogens and herbivores has been observed and evidence of PPOs induction under stress and pathogen attack has been considered. This does not contradict the role of PPOs in biosynthetic reactions in plants. However, one problem is that the role of PPOs in higher plants is probably different from the function in fungi. It has been shown that PPOs in fungi have at least two different functions: the first to defend against pathogens, and the second to act as a pathogenic factor during an attack by fungi on other organisms, including plants. It is still unclear where the reaction of PPOs in plants is directed, although induction of PPOs in attacked cells is often observed. The protective potential of PPOs is still cited by some current studies although the latest works are more critical. PPOs in cucumber seedlings increased significantly after treatment with pectinase extract from the fermentation product of *Penicillium oxalicum* BZH-2002, which means that PPOs are also defensive enzymes (Peng et al., 2004). The experimental data on root PPOs from banana indicate that these enzymes are part of the plant defense system against pests and diseases, including root parasitic nematodes (Wuyts et al., 2006). The clear involvement of root PPOs and dopamine in the resistance of banana to the parasitic nematode *Radopholus similes* has been shown. It has been suggested that although the leaf PPOs of the transgenic hybrid aspen (*Populus tremula* × *Populus alba*) are latent, they are activated by detergents or trypsin of herbivores, and therefore classify this activation during digestion as an adaptive and defense-related feature of poplar PPOs (Wang and Constabel, 2004). There is also evidence that the latent PPOs in early wheat kernel development are activated during later developmental phases, and that enzyme activation is possible by means of limited tryptic digestion in vitro but not by a proteolytic mechanism in vivo (Jukanti et al., 2003). This can be explained by the physicochemical properties of PPOs. It is known that substrate specificity, environmental influences such as pH and temperature, multiplicity, latency, and activators and inhibitors play an important role

in the activity of this group of enzymes (Yoruk and Marshall, 2003). Birch defensive enzyme PPO activity is dependent on temperature. Foliar PPO activity was reduced in leaves with larval twigs at +12°C, but at wounding increased PPO activity at +25°C, suggesting induced susceptibility of birches at low temperatures and induced resistance at higher temperatures (Yang, Ruuhola et al., 2007). Although PPOs are commonly believed to function as an effective defense against herbivory, a study by Barbehenn et al. (2007) questions this function; it demonstrated that in several cases increased PPO levels had no significant effect on insect consumption or growth rates. Yang et al. (2007) suggest that underlying the increase in PPO activity and the decrease in larval growth rate may be $H_2O_2$, which has been shown to accumulate in response to wounding. However, absolute PPO activity decreased with leaf growth due to the dilution effect, whereas the specific activity of PPOs, which has been shown to be defensive against autumnal moth (*Epirrita autumnata*) larvae, did not vary temporally; this might reflect the importance of these enzymes in defense in birches (Yang, Haviola et al., 2007).

The role of PPOs in plant defense against herbivores and pathogens has been hypothesized by many researchers (Jiang and Miles, 1993; Guyot et al, 1995, 1996; Richard-Forget and Gauillard, 1997; Thipyapong et al., 2004; Jukanti 2005; Thipyapong et al., 2007). There is even work showing that PPO is a marker enzyme for defense cells (Schmidt, 1988). No clear evidence of this is yet available, nor in general are the other biological functions of PPOs explained in detail; there are still many questions to be clarified in greater depth. The possible mechanism of the role of PPOs in the plant defense system is unknown, and consequently remains hypothetical, but there have been some attempts to provide evidence. They are based on the fact that some reactive oxygen species PPOs influence $H_2O_2$ production (Jiang and Miles, 1993) and form two equivalents of semiquinone radicals, which can interact with $O_2$ to establish $O_2^-$ molecules (Guyot et al.,1995, 1996). Moreover, the increase in PPO activity in host-pathogen interaction and the binding of *o*-quinones to proteins can induce an antinutritive defense (Dongfeng et al., 2004; Jukanti, 2005). We cannot rule out the possibility that some reactive oxygen species, which may have a function in the systemic signalling network, participate actively in strengthening the plant immunity status. This was shown in some transgenic tomato plants by overexpression of PPOs (Grant and Laoke, 2000; Li and Steffens, 2002; Thipyapong et al., 2007). Some research data indicate the defensive role of plant PPOs in young tissues (Li and Steffens, 2002; Constabel and Ryan, 1998; Jukanti, 2005). This role is regulated by gene cluster 1 in wheat (*Triticum aestivum* L.). PPOs are also known to oxidize phenols and produce fungitoxic quinones, and in this way provide a plant defense against pathogenic fungi (Lattanzio, 2003; Lei et al., 2004). PPO activation in *Vicia faba* by some chemicals, for example uniconazole, is considered an indicator of thylakoid membrane stability (Bekheta et al., 2006). It appears that for plants, catecholases, laccases and cresolases are such indicators, although the molecular activity and generally the mechanism of the activity of these enzymes in plant immunity and the cell protection process is still far from known.

Future research on plant catecholases, laccases and cresolases, and their relation to the same or similar enzymes in natural enemies, will clarify the protection mechanism. However, the protective role of PPOs against herbivores needs more evidence. PPOs play a crucial role in oxidizing phenolic compounds and converting them into reactive defensive molecules (Bi et al., 1997). The molecular mechanisms of this strategy are largely unknown, although it has been suggested that PPOs affect brown coloring and that this in itself plays a role in defending the plant against various biotic and abiotic stresses (Pourcel et al., 2007). Attention should be paid to the role of PPO activities in deciduous tree defenses against herbivores; this has in fact been studied only cursorily, while the role of secondary compounds has been emphasized for decades. It is still not confirmed that PPOs are involved not only in the antagonistic relation between plants and herbivores but, on the other hand, in the mutualistic relationship.

## ACTIVITY IN GERMINATION

PPOs are active compounds during germination of seeds (Taneja and Sachar, 1974; Kocacaliskan et al., 1995; Demeke et al., 2001; Maki and Morohashi, 2006; Dicko et al., 2006). Large seeds have higher PPOs activity than smaller ones, and the activity of PPOs at the beginning of germination is higher than in the following stages (Demeke at al., 2001). There was no change in PPO assay activity for seeds imbibed in water up to 8 h. PPO activity increased in seeds imbibed from 8 to 16 h and then gradually declined with increasing imbibing time.

The presence of PPOs in testas and their induction in endosperm and growing seedlings seem to be potential factors for germination as well as for development and embryo protection. Kocacaliskan et al. (1995) reported that during germination the PPO activity of six plant species seeds did not demonstrate a similar occurrence, but the activity in embryos was higher than in reserve tissues and cotyledons.

Excised coleoptiles and roots of wheat (*Triticum aestivum* L.) have been shown to exhibit high PPO

activity (Taneja and Sachar, 1974). However, research with 50 sorghum varieties yielded data showing that PPOs were not activated in germinated sorghum grains (Dicko et al., 2006). PPO activity in the micropylar region of the endosperm of tomato (*Lycopersicon esculentum*) seeds after radicle protrusion was experimentally demonstrated by Maki and Morohashi (2006). The increased activity of PPOs in the micropylar region was due to an increase in their amounts. It is known that prevailing temperatures and a reduction of the rate of breakdown of protein bodies can have a dual effect on germination. Seeds in natural conditions are frequently exposed to fluctuating temperatures, and the germination behavior of seeds is adjusted to such changes.

Temperature effects cause changes in the phospholipids of membranes during germination, related to events at the ultrastructural level. It can be suggested that exposure to cold during germination either induces the formation of an inhibitor or prevents the formation of an activator that regulates cell metabolism, such as the formation of a key enzyme (Hodson et al., 1986). There is probably a difference in the pattern of PPO occurrence between germinating seeds and seedling organs. It seems that PPOs are more important in seedling growth but there is no empirical evidence of this. It is still unclear what roles light and dark conditions play in PPO activity during germination, although there is evidence that such environmental factors as temperature, day length and humidity influence the activity of PPOs (Shafran et al., 2007). This PPO variability should be investigated in the potential plant response to environmental stress. Additional PPO activity is probably induced in response to light and seedling development. On the other hand, we cannot rule out that during germination and seedling growth plants accumulate more PPOs in their roots for protection against pathogens in case of damage to root tissue. It can be suggested that potential changes in PPO activity during germination as well in plant organs may be a development strategy for plant survival. Future studies of catecholase, laccase and cresolase activity in the germination process offer a promising perspective. The role of these enzymes in different tissues and in processes of germination metabolism, and especially in the testa of seeds, can also clarify many molecular aspects of plant immunity and self-defense as part of the survival strategy. Recent testa studies suggest that enzymatic activity is part of the seed life strategy. Live cells and some other enzymes, such as SDH and AcP, have been found in the testa (Aniszewski, 2005, 2006; Aniszewski et al., 2006). There is evidence that the *Arabidopsis* seed testa contains laccases and no catecholases (Pourcel, 2005). This clearly indicates that the biological role of the *TT10* gene in *Arabidopsis* during seed storage and germination is to create a barrier against pathogens by triggering the formation of antimicrobial quinones.

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