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Non-coordinated response of cytochrome P450-dependent geraniol 10-hydroxylase and NADPH: Cyt C (P-450) reductase in *Catharanthus roseus* hairy roots under different conditions

(With 3 figures)

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Abstract. To obtain basic information about the regulation of the enzyme geraniol 10-hydroxylase, the behavior of this enzyme and its redox partner -the NADPH: Cyt C (P450) reductase- was examined in *C. roseus* hairy roots subjected to various treatments. For several of these treatments, no concerted responses of either enzyme were observed, but in general, reductase was more responsive than G10H. The possible meaning of this finding is discussed.

Key words: Cytochrome P450, geraniol 10-hydroxylase, monooxygenase, P450 reductase, hairy roots, *Catharanthus roseus*.

Abbreviations: geraniol 10-hydroxylase (G10Hase); NADPH cytochrome C (P450) (cyt P450); cytochrome (P450) reductase (CPR); monoterpene indole alkaloids (MIA); abscisic acid (ABA).

Cytochrome P450 is actually a large family of heme-thiolate proteins occurring in microorganisms, yeasts, fungi, insects, plants and vertebrates. To date, plant P450s comprise more than 29 families (Nelson DR; Web homepage, http://drnelson.utmem. edu/biblioD.html; last modified March 2, 1999) and the list grows continuously. Specific plant

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P450s are involved in the biosynthesis of a plethora of substances such as phenylpropanoids, gibberelins, brassinosteroids, jasmonic acid, alkaloids, pigments, terpenes, phytoalexins, etc. [for recent reviews see (6, 7, 34)]. Catalyzes mediated by P450s are diverse and include oxidative dehalogenation, oxidative deamination, aromatic hydroxylation, N-hydroxilation, aliphatic hydroxylation, S- or N-dealkylation and C-C coupling (15, 34). Hydroxylation is the most prominent reaction mediated by plant P450 enzymes. Despite their highly diverse metabolic functions, plant P450-hydroxylases shows a conservative catalytic mechanism. They utilize NADPH to reductively cleave and activate molecular oxygen; one atom is introduced into the substrate (R-H) and the other one is reduced to water (14, 15). Electrons from NADPH are transferred to cyt P450 by a FAD/FMN-containing reductase, the cyt P450 reductase (CPR, E.C. 1.6.2.4). Interaction with this redox partner is an obligatory requirement for biological function of all classical P450 mono-oxygenases.

Mechanisms regulating mammalian P450s have been extensively studied in the last three decades (13). Controls of mammalian P450s are mediated mainly at a transcriptional level but they could be also modulated post-transcriptionally, traslationally and post-traslationally, by protein stabilization or degradation (32). In contrast, regulation of plant P450s has been studied only recently. At present it is known that plant cyt P450s are transcriptionally induced by many factors such as wounding (9, 18), herbicides (29), growth regulators (8, 27, 36), elicitation (2, 27), pathogen attack (32), light (27, 35) and heavy metals (11). They can also be regulated by the developmental state of the plant (28). Information about regulatory mechanisms other than transcriptional control is not yet available for plant P450s. Considerable effort has been spent on the study of plant cyt P450s; much less is known regarding plant P450 reductase modulation, and most work has focused either on cyt P450 regulation or CPR regulation, but independent of each other. Only a few studies have simultaneously analyzed both proteins.

Geraniol 10-hydroxylase (G10H, E.C. 1.14.14), a classical P450 hydroxylase, which catalyzes the C-10 mono-oxygenation of geraniol to 10-hydroxygeraniol, a metabolite *en route* to monoterpene indole alkaloids (MIAs) biosynthesis (21, 22). Many MIAs are used in medicine; pharmacologically, the most important of these are vincristine and vinblastine, two potent cytostatics used as antileukemic agents. In studies carried out *in vitro* on cultures of apocynaceous plants G10Hs catalyze the first committed step in MIA biosynthesis (23, 24, 33). No alkaloids were produced in *in vitro* culture lines where the G10H activity had not been detected, even when other key biosynthetic enzymes were present. When these cultures were transferred to MIAs

induction media, both G10H activity and *de novo* production of MIAs were detected (23, 24). In addition, factors, which increase or decrease G10Hase activity, produce an equivalent effect on MIAs production (33). Although G10H was one of the first P450 mono-oxygenases identified in plants, precise knowledge of its regulation is lacking. This paper reports an examination of G10H and CPR activities (in parallel) in hairy roots of *Catharanthus roseus* (Apocynaceae) upon various treatments credited with stimulating MIA accumulation in *C. roseus* (17, 33, 37).

MATERIALS & METHODS

Hairy root line J1 was previously established by infecting *Catharanthus roseus* seedling roots with *Agrobacterium rhizogenes* (37). The J1 line was maintained by inoculating 0.5 g of fresh tissue in 100 ml of sterile, phytoregulator-free Gamborg's B_5/2 medium (12) plus 3% sucrose. Subcultures, carried out every 14 days were kept in darkness on shakers at 100 rpm and 25°C. Roots on day 24 or day 30 of the culture cycle were treated independently with 5 µM zeatine, 100 µM abscisic acid, 0.1% macerozyme, half strength P-free B_5 medium or half strength B_5 medium with 8% sucrose. All treatments were conducted in triplicate. In addition, an *Aspergillus niger* homogenate (1.8 mg glucose/ml medium) was applied in two flasks of 24 day-old root cultures. Roots on 3% sucrose medium were harvested 96 h after treatment; those on 8% sucrose were harvested after 7 days. Harvested tissue was frozen in liquid N and ground in a mortar. The resultant powder was mixed with 50 mM Tris, 1 mM EDTA at pH 7.5, with 1 mM dithiotreithol (DTT), 1 mM phenylmethylsulphony fluoride (PMSF) and 5 µg/ml leupeptine. The extract was filtered through cheesecloth and centrifuged at 1,500 x g for 15 min. The supernatant was recovered and centrifuged at 100,000 x g for 60 min. The microsomal fraction was resuspended in 50 mM Tris at pH 7.5, 1 mM DTT, 1 mM PMSF, 5 µg/ml leupeptine and 20% (v/v) glycerol. (16)

G10H was assayed as reported by Hallahan et al., (16) and Meijer et al., (24) with some modifications. Briefly, the reaction mixture contained 50 mM potassium phosphate, pH 7.5, 1.2 mM ß-NADPH, 10 µM FAD, 10 µM FMN, 1.2 mM DTT and 68 pmols [1-^{3}H]-geraniol (1 µCi) in a final volume of 25 µl. After 5-10 min, the reaction was stopped with 5 µl of 2 M potassium hydroxide. Cold 10-hydroxygeraniol was added (to a final concentration of 15 mM) as carrier. Monoterpenes were extracted with ethyl acetate and an aliquot spotted on a TLC plate. Authentic standards of monoterpenes were also spotted on the chromatoplates and developed with benzene-acetone-ethyl acetate (2:1:1 v/v/v). Monoterpenes were visualized with iodine vapor; the ra-

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