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Aryltetralin-lignan formation in two different cell suspension cultures of *Linum album*: Deoxypodophyllotoxin 6-hydroxylase, a key enzyme for the formation of 6-methoxypodophyllotoxin

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Dedicated to the memory of Professor Dr. Martin Luckner.

Abstract

Suspension cultures initiated from two different *Linum album* seedlings accumulate either podophyllotoxin (PTOX, 2.6 mg/g DW) or 6-methoxypodophyllotoxin (6MPTOX, 5.4 mg/g DW) as main lignans. Two molecules of coniferyl alcohol are dimerized to pinoresinol which is converted via several steps into deoxypodophyllotoxin (DOP) which seems to be the branching point to PTOX or 6MPTOX biosynthesis. DOP is hydroxylated at position 7 to give PTOX by deoxypodophyllotoxin 7-hydroxylase (DOP7H). In contrast, 6MPTOX biosynthesis is achieved by DOP hydroxylation at position 6 to β-peltatin by the cytochrome P450 enzyme deoxypodophyllotoxin 6-hydroxylase (DOP6H). The following methylation to β-peltatin-A-methylether is catalyzed by β-peltatin 6-*O*-methyltransferase (βP6OMT) from which 6MPTOX is formed by hydroxylation at position 7 by β-peltatin-A-methylether 7-hydroxylase (PAM7H). DOP6H and βP6OMT could be characterized in protein extracts from cell cultures of *L. flavum* and *L. nodiflorum*, respectively, and here in *L. album* for the first time. DOP7H and PAM7H activities could not yet be detected with protein extracts. Experiments of feeding DOP together with inhibitors of cytochrome P450 depending as well as dioxygenase enzymes were performed in order to shed light on the type of DOP7H and PAM7H. Growth parameters and specific activities of enzymes from the phenylpropane as well as the lignan specific biosynthetic pathway were measured during a culture period of 16 days. From the enzymes studied only the DOP6H showed a differential activity sustaining the hypothesis that this enzyme is responsible for the differential lignan accumulation in both cell lines.

Keywords: *Linum album*; Linaceae; Lignan; Podophyllotoxin; 6-Methoxypodophyllotoxin; Deoxypodophyllotoxin 6-hydroxylase; Deoxypodophyllotoxin 7-hydroxylase; β-Peltatin 6-*O*-methyltransferase; β-Peltatin-A-methylether 7-hydroxylase

1. Introduction

According to the definition by IUPAC (Moss, 2000) lignans are a class of secondary metabolites derived from two phenylpropanoid units that are linked by a C–C bond between carbon atoms 8 and 8′ of the side chain carbon atoms. Podophyllotoxin (PTOX) (**5**) is the most important aryltetralin-lignan for human health. It shows cytotoxic and antiviral activities and is used for the treatment of genital warts (*Condylomata acuminata*) caused by the human papilloma virus (Damayanthi and Lown, 1998; Imbert,

Abbreviations: ABT, 1-aminobenzotriazole; CAD, cinnamyl alcohol-dehydrogenase; C4H, cinnamic acid 4-hydroxylase; clot, clotrimazole; cyt *c*, cytochrome *c*; DOP (**1**), deoxypodophyllotoxin; DOP6H, deoxypodophyllotoxin 6-hydroxylase; DOP7H, deoxypodophyllotoxin 7-hydroxylase; 6MPTOX (**4**), 6-methoxypodophyllotoxin; NDA, tetcyclacis; PAL, phenylalanine ammonia-lyase; PAM (**3**), β-peltatin-A-methylether; PAM7H, β-peltatin-A-methylether 7-hydroxylase; βP6OMT, β-peltatin 6-*O*-methyltransferase; 2,4-PCA, 2,4-pyridinedicarboxylic acid; 2,5-PCA, 2,5-pyridinedicarboxylic acid; PTOX (**5**), podophyllotoxin; SAM, S-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; Trinex, trinexapac-ethyl (Pestanal®).

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1998; and literature cited therein). Semisynthetic derivatives of PTOX (**5**) e.g. etoposide, teniposide or etopophos® are used in chemotherapy of cancer. Several *Linum* species accumulate PTOX (**5**) and its derivative 6-methoxypodophyllotoxin (6MPTOX) (**4**) (Weiss et al., 1975; Berlin et al., 1986; Broomhead and Dewick, 1990). In *L. album*, a perennial herbaceous plant growing in Iran and surrounding countries, a PTOX (**5**) content of 0.0005% on a fresh weight basis was observed (Weiss et al., 1975). We have established cell cultures and hairy roots of *L. album* by using plant material from different places in Iran. From 12 lines investigated during the last 10 years, two lines show almost no accumulation of PTOX (**5**) and/or 6MPTOX (**4**), two lines synthesize up to 0.35% PTOX (**5**) and eight lines accumulate up to 0.8% 6MPTOX (**4**) (Empt et al., 2000; Petersen and Alfermann, 2001; Fuss, 2003). The differences in lignan accumulation patterns may be due to the fact that the cultures were initiated from individual seeds with different genotypes collected from the wild. In addition, somaclonal variation can contribute to these differences as well (Phillips et al., 1994; Fuss, 2003; van Fürden et al., 2005). Our hairy root lines of *L. album* accumulate up to 3.5% 6MPTOX (**4**) as main lignan (will be published elsewhere). Taken together this indicates that 6MPTOX (**4**) is the main lignan in *in vitro* cultures of *L. album* and the accumulation of PTOX (**5**) as main compound is the exception. (Empt et al., 2000; Petersen and Alfermann, 2001; Fuss, 2003).

The PTOX (**5**) and 6MPTOX (**4**) biosynthesis starts with the "general phenylpropanoid" pathway (Fig. 1a) where phenylalanine is deaminated by phenylalanine ammonia-lyase (PAL) to give cinnamic acid, hydroxylation by cinnamic acid 4-hydroxylase (C4H) leads to *p*-coumaric acid and after additional steps coniferyl alcohol is formed by cinnamyl alcohol-dehydrogenase (CAD) (Whetten and Sederoff, 1995; Dixon and Reddy, 2003). Two molecules of coniferyl alcohol are coupled to pinoresinol (Fig. 1b). A so called dirigent protein leads to the exclusive formation of (+)-pinoresinol in *Forsythia intermedia* (Davin et al., 1997). In contrast, both enantiomers of pinoresinol can

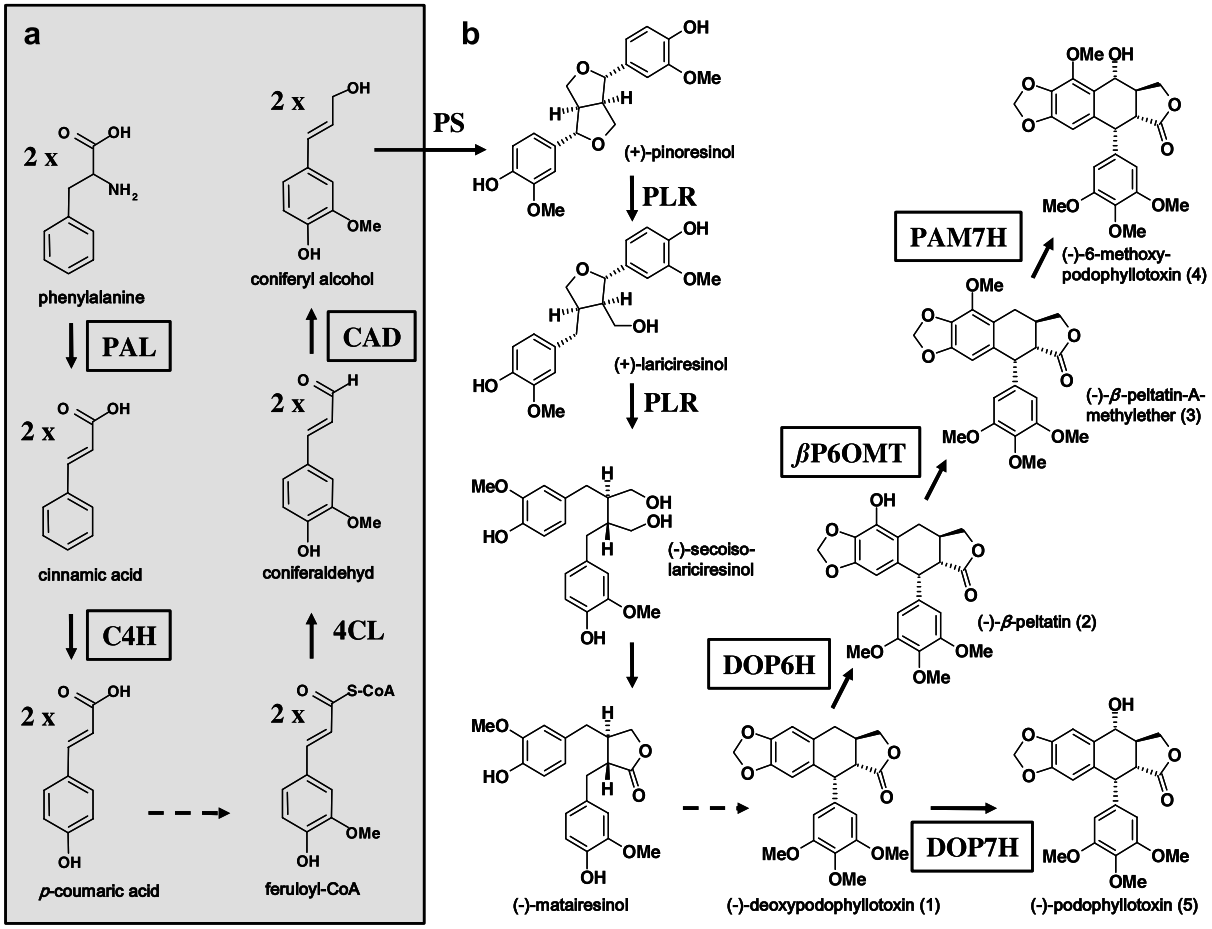

Fig. 1. Hypothetical biosynthetic pathway leading to 6MPTOX and PTOX. (a) Simplified scheme of the general phenylpropanoid pathway; (b) simplified picture of the hypothetical biosynthetic pathway to 6-methoxypodophyllotoxin (6MPTOX, **4**) and podophyllotoxin (PTOX, **5**); two molecules of coniferyl alcohol give one molecule of pinoresinol. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, hydroxycinnamate: CoA ligase; CAD, cinnamyl alcohol-dehydrogenase; PS, pinoresinol synthase; PLR, pinoresinol–lariciresinol reductase; DOP6H, deoxypodophyllotoxin 6-hydroxylase, DOP7H, deoxypodophyllotoxin 7-hydroxylase; βP6OMT, β-peltatin 6-*O*-methyltransferase; PAM7H, β-peltatin-A-methylether 7-hydroxylase.