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Inhibition of phospholipases influences the metabolism of wound-induced benzylisoquinoline alkaloids in *Papaver somniferum* L.

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ABSTRACT

Benzylisoquinoline alkaloids (BIAs) are important secondary plant metabolites and include medicinally relevant drugs, such as morphine or codeine. As the *de novo* synthesis of BIA backbones is (still) unfeasible, to date the opium poppy plant *Papaver somniferum* L. represents the main source of BIAs. The formation of BIAs is induced in poppy plants by stress conditions, such as wounding or salt treatment; however, the details about regulatory processes controlling BIA formation in opium poppy are not well studied. Environmental stresses, such as wounding or salinization, are transduced in plants by phospholipid-based signaling pathways, which involve different classes of phospholipases. Here we investigate whether pharmacological inhibition of phospholipase A_2 (PLA_2, inhibited by aristolochic acid (AA)) or phospholipase D (PLD; inhibited by 5-fluoro-2-indolyl des-chlorohalopemide (FIPI)) in poppy plants influences wound-induced BIA accumulation and the expression of key biosynthetic genes. We show that inhibition of PLA_2 results in increased morphinan biosynthesis concomitant with reduced production of BIAs of the papaverine branch, whereas inhibition of PLD results in increased production of BIAs of the noscapine branch. The data suggest that phospholipid-dependent signaling pathways contribute to the activation of morphine biosynthesis at the expense of the production of other BIAs in poppy plants. A better understanding of the effectors and the principles of regulation of alkaloid biosynthesis might be the basis for the future genetic modification of opium poppy to optimize BIA production.

1. Introduction

Opium poppy is one of the world́s most prominent medicinal plants because it contains therapeutically important benzylisoquinoline alkaloids (BIAs) (Beaudoin and Facchini, 2014). While there are more than 2500 structurally different natural BIAs, which are produced mainly by plants of the order Ranunculales, morphine is only produced by opium poppy species, such as *Papaver somniferum* or *Papaver setigerum* (Ziegler et al., 2005). Even though several protocols for the chemical synthesis of morphine and its derivatives have been published (Rinner and Hudicky, 2012) and their production *via* microbial biomanufacturing seems to be promising (Fossati et al., 2015; Thodey et al., 2014), *de novo* synthesis and chemical conversion of these compounds are not always economical owing primarily to the occurrence of five chiral centers in the morphinan backbone structure (Wijekoon and Facchini, 2012). Thus, despite substantial efforts, the extraction of morphinans

Abbreviations: AA, aristolochic acid; BBE, berberine bridge enzyme; BIA(s), benzylisoquinoline alkaloid(s); CFS, cheilanthifoline synthase; CNMT, (*R,S*) coclaurine-*N*-methyltransferase; CODM, codein-*O*-demethylase; COR, codeinone reductase; DMSO, dimethyl sulfoxide; DRR, 1,2-dehydroreticuline reductase; DRS, 1,2-dehydroreticuline synthase; FAA(s), free fatty acid (s); FIPI, 5-fluoro-2-indolyl des-chlorohalopemide; 4-HPAA, 4-hydroxyphenylacetaldehyde; 4-HPP, 4-hydroxyphenylpyruvate; JA, jasmonic acid; MJ, methyl jasmonate; MAPK, mitogen-activated protein kinase; MRM, multiple reaction monitoring mode; NCS, norcoclaurine synthase; NMCH, (*S*)-*N*-methylcoclaurine-3-hydroxylase; N7-OMT, norreticuline-7-*O*-methyltransferase; 4-OMT, (*R,S*)-3'-hydroxy-*N*-methylcoclaurine-4'-*O*-methyltransferase; 6-OMT, (*S*)-norcoclaurine-6-*O*-methyltransferase; 7-OMT, (*R,S*)-reticuline-7-*O*-methyltransferase; PA, phosphatidic acid; P6H, protopine-6-hydroxylase; PLA_2, phospholipase A_2; PLD, phospholipase D; *Ps*PLA_2, phospholipase A_2 from *Papaver somniferum* L; *Ps*PLD, phospholipase D from *Papaver somniferum* L; RT-qPCR, *real-time* quantitative polymerase chain reaction; SalAT, salutaridinol-7-*O*-acetyltransferase; SalR, salutaridine reductase; SalSyn, salutaridine synthase; SIPK, salicylic acid-induced protein kinase; STSy, stylopine synthase; TNMT, tetrahydroprotoberberine-*N*-methyltransferase; T6ODM, thebaine-6-*O*-demethylase; TYDC, tyrosine/DOPAdecarboxylase; TyrAT, tyrosine aminotransferase; WIPK, wound-induced protein kinase

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from opium poppy (*Papaver somniferum* L., Papaveraceae) is currently the only commercial source for morphine and codeine, and the default source for semi-synthetic derivatives, such as oxycodone and naltrexone (Beaudoin and Facchini, 2014).

The biosynthesis of BIAs in opium poppy starts with *L*-tyrosine, which is converted to (*S*)-norcoclaurine as the first committed BIA intermediate. The enzymes (*R,S*)-coclaurine-*N*-methyltransferase (CNMT) and (*R,S*)-3'-hydroxy-*N*-methylcoclaurine-4'-*O*-methyltransferase (4-OMT) are key subsequent steps mediating the conversion of (*S*)-norcoclaurine to (*S*)-reticuline, which serves as the starting point for several further branches of BIA metabolism. (*S*)-reticuline can be converted by the berberine bridge enzyme (BBE) to (*S*)-scoulerine, an intermediate in the noscapine branch. Alternatively, (*S*)-reticuline can be converted in several steps to papaverine, involving the (*R,S*)-reticuline-7-*O*-methyltransferase (7-OMT), forming the papaverine branch. The biosynthesis of morphinans represents the third branch, which requires the stereochemic conversion of (*S*)-reticuline to (*R*)-reticuline. The morphinan branch involves the production of salutaridine, thebaine and oripavine, and ultimately leads to the production of codeine and morphine. One of the key steps in this branch is mediated by salutaridinol-7-*O*-acetyltransferase (SalAT). While the intermediates mentioned cover the main branches of BIAs metabolism in poppy plants, the transcript abundance corresponding to the enzymes mentioned might indicate possible targets for regulation.

In planta, BIAs act in defence reactions against herbivores and pathogens and can inhibit the growth of competitor plants (allelopathy) (Evans, 2009). BIAs have also been reported to act in the adaptation of opium poppy plants to environmental and stress conditions, including wounding (Mishra et al., 2013; Morimoto et al., 2001). Various studies indicate that BIA biosynthesis in poppy is regulated at different levels, including hormonal and transcriptional regulation (Beaudoin and Facchini, 2014), in line with their biological functions. From other plant model systems, such as *Arabidopsis*, it is known that wounding stress triggers responses mediated by phospholipid-derived signaling molecules (Munnik, 2001; Mosblech et al., 2008). Therefore, we analyzed possible links between phospholipase- and phospholipid-based signaling and BIA production in poppy plants. The transcript levels for poppy phospholipase A_2 (*Ps*PLA$_2$) and phospholipase D1 (*Ps*PLD1) were previously shown to be wound-induced in early stages after wounding, and the encoded enzymes have been characterized (Jablonická et al., 2016; Lerchner et al., 2005). While the biochemical characteristics of relevant phospholipases from opium poppy (*Ps*PLA$_2$, phospholipase A_2 from *Papaver somniferum* L.; *Ps*PLD1, phospholipase D1 from *Papaver somniferum* L.) have previously been described and the genes cloned and identified (Jablonická et al., 2016; Lerchner et al., 2005; Obložinský et al., 2003), a molecular approach to perturb phospholipid signaling in opium poppy is currently not feasible. However, pharmacological inhibitors for phospholipases are well characterized for their use in plants. These inhibitors, such as aristolochic acid (AA) inhibiting phospholipase A_2 (PLA$_2$), or 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) inhibiting phospholipase D (PLD), have successfully been used in a number of plant studies (Gao et al., 2013; Gardiner et al., 2008; Su et al., 2009). In our work, we used a pharmacological approach to inhibit PLA$_2$ or PLD in opium poppy plants to identify new molecular players involved in the control of alkaloid accumulation in intact or wounded opium poppy. A better understanding of these new players and principles of regulation of BIA biosynthesis might be the basis for the future genetic modification of opium poppy to optimize the production of BIAs, as was already reported for other secondary plant products (Fazal et al., 2016; Kamalipourazad et al., 2016; Yendo et al., 2010).

Table 1
Summary of applied phospholipaseś inhibitors and their function.

Inhibitor/Control	Function	Concentration
Hoagland solution	Control	#
DMSO	second control	0.1% (v/v)
AA	inhibition of PLA$_2$	50 µM
FIPI	inhibition of PLD	100 nM

2. Material and methods

2.1. Growing of plant material

Papaver somniferum L. seedlings were sown on a moistened perlite and grown in the dark in a climate growth-chamber at 20–22 °C and 60–70% relative humidity.

On the fifth day of post-imbibition, seedlings were harvested, immediately frozen in liquid nitrogen and stored at −80 °C (later used for total RNA or lipid extraction, respectively).

To grow hydroponic poppies, 5-days-old seedlings were transported into ½ Hoagland culture solution (Hoagland and Arnin, 1950) and were growing at 20–22 °C and 60% relative humidity under a photoperiod of 16 h light/8 h dark cycle with a light intensity of 7 000 lx. After two days, the concentration of culture solution was enhanced to 1 and plant's roots were supplied with oxygen. The culture solution has been changed two times per week. 35-days-old poppy plants were transferred into 200 ml of culture solution with addition of inhibitors, basal culture solution (control group) or Hoagland solution with 0.1% DMSO (second control) (Table 1). After 24 h of incubation, one third of the plants from each group were harvested, leaves of the remaining plants were mechanically wounded and additionally incubated for 3 or 5 h, respectively. The plant material was used for RT-qPCR analysis and for estimation of alkaloid content using LC–MS.

2.2. Real-time quantitative PCR

Total RNA was extracted from various biological samples. 5 µg of total RNA was used as a template for cDNA synthesis. Reaction setup for the RT-qPCR (total reaction volume : 15 µl): 7.5 µl 2 × Maxima SYBR Green qPCR Master Mix (final concentration 1 ×), 3 µl of Mastermix Primer (5 µM each − final concentration 1 µM each), 2 µl template cDNA, 2.5 µl dsH_2O. As a housekeeping gene β-actin was chosen. All RT-qPCR assays were performed on Real-time PCR Cyclers: Rotor-Gene Q 2plex or Applied Biosystems 7300 in a program: 96 °C 10 min, 96 °C 10 s, 65 °C 15 s, 72 °C 20 s.

2.3. Alkaloid analysis

2.3.1. Alkaloid extraction

Poppy leaves were frozen in liquid nitrogen and ground to a fine powder. Approximately 50 mg of plant tissues was used for alkaloid extraction. Exact weight of each sample was measured. The plant tissues were subjected to extraction with 300 µl of 50% (v/v) methanol. 100 ng of dextromethorphan (Sigma Aldrich, Germany) as internal standard was added to each sample. After 20 min incubation by shaking at room temperature, samples were centrifuged at 10 000 × *g* for 10 min. Eluates were transferred to a new 1.5 ml microtube and diluted 1:10 with 50% (v/v) methanol to final volume of 200 µl. After centrifugation at 10 000 × *g* for 10 min the samples were transferred to autosampler vials for the LC–MS/MS analysis.

2.3.2. LC-ESI–MS analysis

Separations were performed on a Nucleoshell RP18 column (50 × 3 mm, particle size 2.7 µm; Macherey-Nagel, Germany) at 30 °C using an Agilent 1290 Infinity HPLC system. Eluents A and B were