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Identification of CYP82E21 as a functional nicotine *N*-demethylase in tobacco flowers

Verena Liedschulte*, Joanne Deborah Schwaar, Hélène Laparra, Aline Vuarnoz, Bérangère Philippon, Nicolas Bakaher, Nicolas Sierro, Lucien Bovet, Gerhard Lang, Simon Goepfert

PMI R&D, Philip Morris Products SA, (part of Philip Morris International Group of Companies), Neuchâtel, Switzerland

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ABSTRACT

In the tobacco plant, nicotine *N*-demethylase enzymes (NND) belonging to the cytochrome P450 family catalyse the conversion of nicotine to nornicotine, the precursor of the carcinogenic tobacco-specific *N*-nitrosamine, *N*-nitrosonornicotine. To date three demethylase genes, namely *CYP82E4*, *CYP82E5* and *CYP82E10*, have been shown to be involved in this process, while the related *CYP82E2* and *CYP82E3* genes are not functional. We have identified a further gene named *CYP82E21* encoding a putative nicotine *N*-demethylase closely related to the *CYP82E* genes. The *CYP82E21* gene was found in all *Nicotiana tabacum* cultivars analysed and originates from the tobacco ancestor *Nicotiana tomentosiformis*. We show that, in contrast to all other previously characterized *NND* genes, *CYP82E21* is not expressed in green or senescent leaves, but in flowers, more specifically in ovaries. The nicotine *N*-demethylase activity of CYP82E21 was confirmed by ectopic expression of the coding sequence in a tobacco line lacking functional *CYP82E4*, *CYP82E5* and *CYP82E10* genes, resulting in an eightfold increase of nicotine demethylation compared to the control plants. Furthermore, nornicotine formation can be reduced in ovaries by introducing a *CYP82E21*-specific RNAi construct. Together, our results demonstrate that the *CYP82E21* gene encodes a functional ovary-specific nicotine *N*-demethylase.

1. Introduction

The pyridine alkaloids nicotine, nornicotine, anatabine and anabasine are found in many species of the genus *Nicotiana* at varying relative concentrations. The alkaloid fraction of the commercially most relevant plant, tobacco (*Nicotiana tabacum*), consists predominantly (>90%) of nicotine (Eich, 2008).

Due to their pronounced neurotoxic activity, these alkaloids serve as an efficient protection of the plants against herbivores. In addition, their presence in floral rewards has been shown to modulate pollinator behaviour. In line with their ecological role as antifeedants, the production of alkaloids in the roots and their accumulation in the shoot increase upon leaf damage (Baldwin, 1988; Baldwin and Ohnmeiss, 1993; Halpern et al., 2010). In tobacco production, the biosynthesis of nicotine is also induced by the common agricultural practice of "topping" (i.e., the removal of the flowering head) (Qi et al., 2012).

In cultivated tobacco, alkaloids with a secondary amine moiety such as nornicotine and anabasine are undesired as they are direct precursors to carcinogenic tobacco-specific *N*-nitrosamines (TSNAs) (reviewed by Hoffmann et al., 1994). TSNA formation mostly takes place during the curing of the tobacco leaf (Andersen et al., 1987; Burton et al., 1994), a drying process in which the tobacco leaf is changed both physically and chemically, and is essential for both tobacco aroma and flavour. During this curing process nitrate is microbially reduced to nitrite which may react then with alkaloids to yield nitrosamines. Further quantities of TSNAs may be formed during tobacco pyrolysis (Moldoveanu and Borgerding, 2008).

Besides its role as a precursor to *N*-nitrosonornicotine (NNN),

* Corresponding author.
E-mail addresses: Verena.Liedschulte@pmi.com (V. Liedschulte), Joanne.Schwaar@pmi.com (J.D. Schwaar), Helene.Laparra@pmi.com (H. Laparra), Aline.Vuarnoz@pmi.com (A. Vuarnoz), Berangere.Philippon@pmi.com (B. Philippon), Nicolas.Bakaher@pmi.com (N. Bakaher), Nicolas.Sierro@pmi.com (N. Sierro), Lucien.Bovet@pmi.com (L. Bovet), Gerhard.Lang@pmi.com (G. Lang), Simon.Goepfert@pmi.com (S. Goepfert).

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nornicotine itself may cause adverse physiological effects in humans by inducing aberrant glycation of proteins and covalent binding to commonly used steroid drugs (Dickerson and Janda, 2002).

In tobacco, nornicotine is formed by oxidative demethylation of nicotine. This conversion of nicotine to nornicotine is catalysed by *N*-demethylase enzymes (NND) belonging to the family of cytochrome P450 monooxygenases (CYP). Several genes coding for such NNDs have been identified in tobacco, of which three NNDs have been shown to be involved in nornicotine formation in tobacco, namely CYP82E4, CYP82E5 and CYP82E10 (Gavilano and Siminszky, 2007; Lewis et al., 2010; Siminszky et al., 2005). Of these NNDs, CYP82E4 contributes most to nornicotine formation and differs from CYP82E5 and CYP82E10. Indeed, *CYP82E4* is specifically expressed in senescent leaves and plays a major role in nicotine *N*-demethylation during curing (Chakrabarti et al., 2008). Interestingly, the fraction of the total alkaloids in tobacco leaves that is nornicotine can rise from normally <5% to as much as 98% due to an unstable "converter locus" that stimulates transcript accumulation of *CYP82E4* (Gavilano et al., 2006; Mann et al., 1964). Two further related genes identified in tobacco, *CYP82E2* and *CYP82E3*, do not code for functional NNDs due to point mutations (Chakrabarti et al., 2007; Gavilano et al., 2007; Siminszky et al., 2005).

In order to decrease the NNN content in tobacco, it is possible to reduce the plant nitrate content or its metabolisation to nitrite by modifying the curing process or by microbial treatment (Lu et al., 2016; Wei et al., 2014). However, the knowledge of the nornicotine biosynthesis pathway in tobacco provides an alternative strategy the targeting of the three identified NND genes *CYP82E4*, *CYP82E5* and *CYP82E10* by RNA interference or by introgression of non-functional variant forms. RNAi-induced silencing of *CYP82E4* and its close homologues efficiently reduced nicotine to nornicotine conversion to about 0.8% (Gavilano et al., 2006) and consequently leads to cured tobacco with reduced NNN levels (Lewis et al., 2008). Knockout mutations in all three genes likewise reduced conversion of nicotine to nornicotine to about 0.5% (Lewis et al., 2010). The remaining nornicotine level suggests that either the presence of further functional NND genes or a contribution of other, hitherto unidentified, processes to nornicotine formation exist.

In this study, we report the identification of a novel member of the nicotine *N*-demethylase family, its temporal and organ-specific expression pattern, and its contribution to nicotine demethylation in different plant organs.

2. Results

2.1. The nicotine N-demethylase CYP82E21 is most closely related to CYP82E4

During a genomic DNA sequencing effort aimed at building a reference tobacco genome, we identified a novel member of nicotine *N*-demethylases that is very closely related to the described *CYP82E* genes and that was termed *CYP82E21* according to David Nelson's classification (Nelson, 2006). The *CYP82E21* gene could be identified in all *N. tabacum* varieties analysed. Its sequence was confirmed by PCR amplification and Sanger sequencing.

The *N. tabacum CYP82E21* gene is of *Nicotiana tomentosiformis* origin since related sequence and transcripts have been observed in *N. tomentosiformis*, but not in *Nicotiana sylvestris* (Sierro et al., 2013). At the protein sequence level, CYP82E21 is most closely related to CYP82E4 (94% identity) and to CYP82E3 and CYP82E2 (94 and 93% identity, respectively) and it shares 91% identity with CYP82E5 and CYP82E10 (Fig. 1 and Fig. S1).

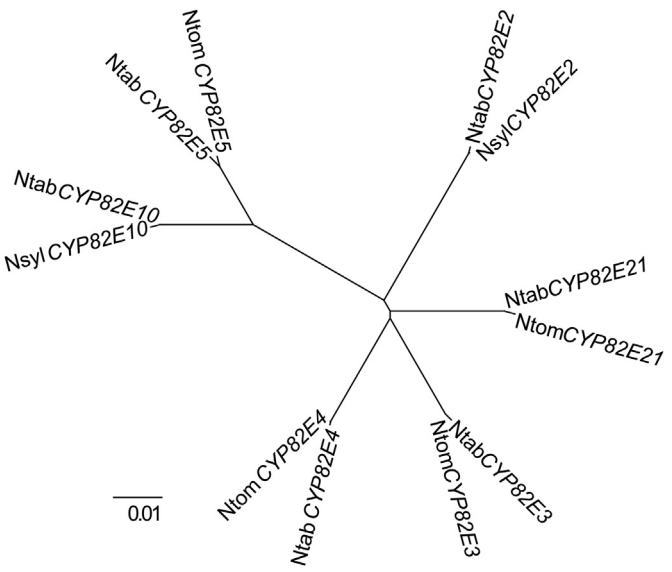

Fig. 1. Unrooted tree of *CYP82E21* and related *CYP82E* coding sequences, generated by the neighbour-joining method (Ntab: *N. tabacum*; Ntom: *N. tomentosiformis*; Nsyl: *N. sylvestris*).

2.2. CYP82E21 is not expressed in leaves but in flowers

As previously reported, *CYP82E4* and *CYP82E2* are strongly induced upon air-curing, whereas *CYP82E5*, *CYP82E10* and *CYP82E3* are already expressed in green tobacco leaves (Chakrabarti et al., 2007).

The *CYP82E2* gene was inherited from *N. sylvestris* where it is still functional and leads to almost complete metabolism of nicotine to nornicotine during curing, whereas the *CYP82E3* gene is derived from *N. tomentosiformis* where it is likewise still functional and leads to high conversion of nicotine to nornicotine in green leaves (Chakrabarti et al., 2007). The *CYP82E2* and *CYP82E3* genes in *N. tabacum* are not functional nicotine *N*-demethylases due to point mutations leading to amino acid exchange in crucial regions of the enzyme (Chakrabarti et al., 2007; Siminszky et al., 2005; Fig. S1).

To determine the function of CYP82E21 and its role in nicotine conversion, the expression of *CYP82E21* and of the other functional *CYP82E* genes was analysed. For this purpose, different organs of greenhouse grown *N. tabacum* var. TN90 were harvested, RNA was extracted and analysed via quantitative PCR. The PCR products were sequenced to confirm the specificity of the reaction.

The results are shown in Fig. 2 and confirm the expression of *CYP82E5* and *CYP82E10* in green leaves (Gavilano et al., 2007). They were also expressed in all other organs that were tested. *CYP82E5* and *CYP82E10*, inherited from *N. tomentosiformis* and from *N. sylvestris*, respectively, are most likely homeologs as they show a very high sequence identity and a similar expression pattern. Due to the sequence identity of *CYP82E21* with *CYP82E4*, which is like *CYP82E21* of *N. tomentosiformis* origin, we expected a similar expression pattern for *CYP82E4* and *CYP82E21*. *CYP82E4* is reported to be expressed during senescence and also to show higher expression in mature, compared to immature flowers (Chakrabarti et al., 2008), an observation which we also made. While *CYP82E4* and *CYP82E21* were both expressed in flowers, *CYP82E21* was not expressed at detectable levels in leaves whereas *CYP82E4* showed expression exclusively in senescent leaves. *CYP82E4* and *CYP82E21* were both expressed in roots, albeit to an extremely low extent.