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Unravelling the architecture and dynamics of tropane alkaloid biosynthesis pathways using metabolite correlation networks

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ABSTRACT

The tropane alkaloid spectrum in *Solanaceae* is highly variable within and between species. Little is known about the topology and the coordination of the biosynthetic pathways leading to the variety of tropine and pseudotropine derived esters in the alkaloid spectrum, or about the metabolic dynamics induced by tropane alkaloid biosynthesis stimulating conditions. A good understanding of the metabolism, including all ramifications, is however necessary for the development of strategies to increase the abundance of pharmacologically interesting compounds such as hyoscyamine and scopolamine. The present study explores the tropane alkaloid metabolic pathways in an untargeted approach involving a correlation-based network analysis. Using GC–MS metabolite profiling, the variation and co-variation among tropane alkaloids and primary metabolites was monitored in 60 *Datura innoxia* Mill. individuals, of which half were exposed to tropane alkaloid biosynthesis stimulating conditions by co-culture with *Agrobacterium rhizogenes*. Considerable variation was evident in the relative proportions of the tropane alkaloids. Remodeling of the tropane alkaloid spectrum under co-culture with *A. rhizogenes* involved a specific and strong increase of hyoscyamine production and revealed that the accumulation of hyoscyamine, 3-tigloyloxy-6,7-epoxytropane, and 3-methylbutyryloxytropane was controlled independently of the majority of tropane alkaloids. Based on correlations between metabolites, we propose a biosynthetic origin of hygrine, the order of esterification of certain di-oxygenated tropanes, and that the rate of acetoxylation contributes to control of hyoscyamine production. Overall, this study shows that the biosynthesis of tropane alkaloids may be far more complex and finely controlled than previously expected.

1. Introduction

A few genera of the plant family *Solanaceae*, including *Datura*, *Hyoscyamus*, *Duboisia*, *Atropa* and *Scopolia*, produce a wide range of tropane alkaloids in their roots. These secondary metabolites are mainly esters of tropine (tropan-3-α-ol) and pseudotropine (tropan-3-β-ol). The tropine ester, hyoscyamine, and its 6,7-β-epoxide, scopolamine, are medicinally important due to their anticholinergic activities on the central nervous system (Akramian et al., 2008). Despite the fact that the biosynthetic pathway of hyoscyamine and scopolamine has been thoroughly studied, the knowledge of several biosynthetic steps remains incomplete and only a few tropane alkaloid biosynthetic genes have been discovered (Bedewitz et al., 2014; Humphrey and O'Hagan, 2001; Jirschitzka et al., 2013). For example, to date no acyltransferase involved in the transfer of the acidic moiety to tropine or pseudotropine has been cloned. In a protein extract from *Datura stramonium*, a fraction containing pseudotropine acylating activity was separated from a fraction that was enriched in tropine acylating activity and was shown to act on a wide range of aliphatic acyl-CoA donors (Boswell et al., 1999; Rabot et al., 1995; Robins et al., 1994, 1991a). It has been proposed therefore that the relative availability of the different acyl-CoA donors could be the main factor determining the extent to which each tropine or pseudotropine ester accumulates (Boswell et al., 1999; Rabot et al., 1995; Robins et al., 1991a). Nevertheless, in most species the spectrum of alkaloids is dominated by tropine-derived esters and a pure tropine acylating activity has never been reported (Robins et al., 1994). Thus, much remains unknown about the factors influencing the distribution of biosynthetic flux throughout the network of tropine

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and pseudotropine ester production. Similarly, the physiological function of the diverse tropane alkaloids in plants has not been elucidated. Could there be a complex mechanism that governs the variation in the tropane alkaloid spectrum in *Solanaceae* within and between species, and in response to interactions with the environment? Berkov and Zayed (2004) reported that *Datura innoxia* Mill. cultivated in tropical (Egypt) and continental (Bulgaria) climates showed strong variation in the levels of 38 tropane alkaloids. Hairy root cultures, induced from several *Solanaceae* species by *Agrobacterium rhizogenes* transformation, accumulate higher concentrations of tropane alkaloids than parent roots, and treatment with methyl jasmonate increases this effect (Kang et al., 2004; Maldonado-Mendoza et al., 1992). A substantial variation of relative proportions of tropane alkaloid levels was also reported within species in the petioles of 1000 individual plants of the genera *Datura*, *Scopolia* and *Hyoscyamus* grown under identical conditions, and in their hairy root cultures initiated by *A. rhizogenes* transformation (Parr et al., 1990).

The complex variation in the tropane alkaloid profile, and more specifically the co-variation of different tropane alkaloids, is a potential source of valuable information. Correlations between metabolites arise from fluctuations in (at least some) metabolite levels caused by internal or external factors, in combination with the effect of many biochemical reactions and regulatory interactions, and constitute therefore a global property of the system. In most cases, the complex origin of these correlations does not allow a straightforward interpretation in terms of the underlying biochemical pathways (Steuer, 2006). Nevertheless, large-scale non-targeted metabolomic analyses in combination with a correlation-based network analysis of the resulting metabolic profiles may reveal metabolites or pathways that are subjected to similar control mechanisms. Differences in metabolite–metabolite correlations between different conditions may highlight the biochemical pathways that are affected by the studied conditions and reveal key components in the metabolic regulation. This approach has recently proved useful in the reconstruction of metabolic pathways (Krumsiek et al., 2011), in the identification of metabolic pathway responses to changing environments (Fukushima and Kusano, 2014; Kotze et al., 2013), in the identification of pathways that are involved in the regulation of biological processes (Albrecht et al., 2014), and in the identification of genetic regulators for future breeding and metabolic engineering strategies (Hu et al., 2014). Knowledge of the biosynthetic pathways and control mechanisms is crucial for the development of genetic engineering strategies to increase the yield of certain pharmaceutically active compounds, as well as speculations of cause and effect of genetic transformations (Jenner, 2003). To shed light on the topology and dynamics of the tropane alkaloid biosynthetic pathways, here we conduct a comprehensive comparison of the accumulation dynamics of the metabolites in roots of a large number of *D. innoxia* individuals, hydroponically cultured under conditions that differentially affect the production of tropane alkaloids.

2. Results

2.1. Differential tropane alkaloid and primary metabolite accumulation

In order to study the relations between the levels of the different tropane alkaloid biosynthesis intermediates and products, as well as the primary metabolite levels, GC–MS based metabolite profiling was conducted on root extracts of 60 hydroponically cultured *D. innoxia* individuals, half of which were treated with addition of *A. rhizogenes* to the culture medium. The roots of the treated plants showed a strong proliferation, which is typical for the hairy root disease caused by *A. rhizogenes* infection (Fig. 1). Based on

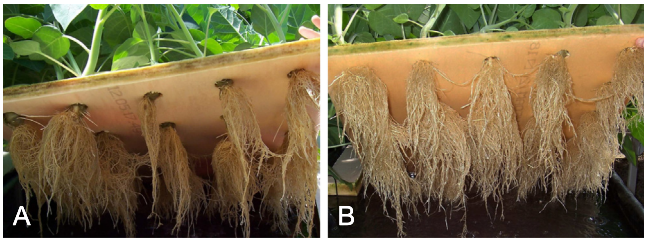

Fig. 1. Roots of *D. innoxia* cultivated in hydroponic culture without *A. rhizogenes* co-culture (“control plants”, A) and with *A. rhizogenes* co-culture (“treated plants”, B).

fragmentation spectra and relative retention times of alkaloids previously reported for *Datura* spp., 47 compounds were annotated as alkaloids (Table 1). Many tropane esters (**10**, **11**, **15**, **17**, **18**, **19**, **23**, **24**, **40**, **41**) appear as double peaks in the GC chromatogram and show identical mass spectra. Although the stereochemistry of these compounds cannot be established solely by MS data, it is generally expected that these peaks are isomeric pseudotropine and tropine esters, as was shown for 3-acetoxytropanes and 3-tigloyloxytropanes (**15** and **17**) (Witte et al., 1987). Two isomeric forms of 3-tigloyloxy-6,7-epoxytropane (**18** and **19**), which was previously reported as a single isomer in *D. stramonium* roots (El Bazaoui et al., 2011), were found here. Out of 47 alkaloids detected, the abundance of 27 compounds was significantly higher in treated plants than in control plants (Table 1). The increases of the mean peak intensities upon co-culturing *A. rhizogenes* varied from less than threefold for 16 alkaloids, to more than 14-fold for hyoscyamine. Of the 67 polar compounds with known identity, none showed a significantly different accumulation between control and treated plants (Table S1). However, an organic acid that was not fully elucidated (**87**) was found to be present in significantly higher amounts in treated plants. None of the analyzed alkaloids or primary metabolites showed a significantly lower accumulation in treated plants. The relative proportions of different tropane alkaloids were highly variable in the control plants. For example, the proportion of 3α-tigloyloxytropane (**15**) to hyoscyamine (**36**) levels, measured based on the intensity of the common base peak at *m/z* 124 in their fragmentation spectrum, varied from 0.2 to 1.6 in control plants. In contrast, the alkaloid profiles of the treated plants were generally dominated by hyoscyamine (**36**). The degree of variability in accumulation of each metabolite in control and treated plants was expressed in the form of the coefficient of variation (Table 1, Table S1). The coefficient of variation of more than half of the alkaloids, including hyoscyamine (**36**) and scopolamine (**43**), decreased when the plants were treated. Among the tropane alkaloids, only 4′-hydroxylittorine (**44**) and 3-(3′-acetoxytropoyloxy)tropane (**42**) had a much higher coefficient of variation in treated plants than that in control plants. Most primary metabolites also showed a decreased coefficient of variation in the treated plants.

2.2. Overall metabolite correlation networks

To reveal metabolites with similar accumulation profiles, all pairwise correlations were calculated between metabolite levels within the dataset of 115 compounds, in each of the two experimental groups of *D. innoxia* plants (control and treated plants). Significant correlations were visualized in networks, where each node represents a metabolite and each edge represents a significant correlation (see Experimental section). There were 1023 and 929 pairs of significant positive correlations, and 332 and 213 pairs of significant negative correlations, in control and treated plants, respectively. The overall correlation networks (Fig. S1) showed that, in both control and treated plants, metabolites that belonged

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