Genetic analysis of alkaloid composition and translocation in wild *Nicotiana* species

野生タバコ種におけるアルカロイドの組成と長距離輸送の遺伝学的解析

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Graduate School of Biological Sciences　Doctoral Thesis Abstract

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Title	**Genetic analysis of alkaloid composition and translocation in wild *Nicotiana* species**		

Abstract

Nicotine and related pyridine alkaloids are produced in the root of *Nicotiana* species and translocated through the xylem to the aerial parts of the plants, where they function as part of chemical defense against insect herbivory. Although a few tobacco transporters have recently been reported to take up nicotine into the vacuole from the cytoplasm, or into the cytoplasm from the apoplast, it is not known how long-distance translocation of tobacco alkaloids between organs is controlled. Two closely related wild *Nicotiana* species, *N. alata* and *N. langsdorffii*, differ in their translocation ability and alkaloid composition. I show here that *N. langsdorffii* produces predominantly nicotine in the root, and accumulates exclusively nicotine in the leaf. In contrast, *N. alata* produces mainly nicotine and smaller amounts of nornicotine and anatabine in the root, but does not accumulate any tobacco alkaloids in the leaf, thus representing a non-translocation phenotype in *N. alata*. Three experiments, namely reciprocal grafting, xylem sap collection and hairy root culture, confirmed that the root organ of *N. alata* is deficient in alkaloid export. Interestingly, genetic analysis involving interspecific hybrids between *N. alata* and *N. langsdorffii* and their self-crossed or back-crossed progeny showed that the non-translocation phenotype is dominant over the translocation phenotype. These results indicate that a mechanism to retain tobacco alkaloids within the root organ has evolved in *N. alata*, which may represent an interesting strategy to control the distribution of secondary products within the whole plant.

I also examined nornicotine formation and the possible involvement of *CYP82E* genes in these two *Nicotiana* species. Nornicotine is found abundantly in *N. alata*, but is absent in *N. langsdorffii*. Nornicotine is known to be derived from nicotine by a nicotine *N*-demethylase, a CYP82E-family monooxygenase. I identified four *CYP82E* genes in *N. alata* that encoded functional nicotine *N*-demethylases, whereas two *CYP82E* genes in *N. langsdorffii* were observed to be inactivated by different molecular mechanisms. One *N. langsdorffii CYP82E* gene is weakly expressed but contains a single nucleotide deletion in the first exon, resulting in the premature termination of translation and thus producing a truncated protein. Another *N. langsdorffii CYP82E* gene, which encodes a functional enzyme, is not expressed at all. Expression analysis of interspecific F_1 hybrids between *N. alata* and *N. langsdorffii* suggested that the absence expression of this otherwise functional *CYP82E* gene in *N. langsdorffii* is due to a defect in the *cis*-regulatory activity. These results indicate that the non-functionalization of *N. langsdorffii CYP82E* genes by premature translational termination and transcriptional inactivation plays a key role in the elimination of nornicotine in *N. langsdorffii*. Genetic analysis further suggested that the nornicotine-forming phenotype is controlled by a single semi-dominant locus, and that duplicated *CYP82E* genes in either species are genetically linked. My study provides genetic evidence that *CYP82E* genes are solely responsible for nornicotine formation in these wild *Nicotiana* species.

CONTENTS

CONTENTS (continued)

CONTENTS (continued)

LIST OF FIGURES

LIST OF TABLES

LIST OF SUPPLEMENTARY FIGURES

LIST OF SUPPLEMENTARY TABLES

CHAPTER I

Root-to-shoot translocation of alkaloids is dominantly suppressed in *Nicotiana alata*

Introduction

Tobacco alkaloids are natural chemical defense compounds, and nicotine is a major toxic alkaloid synthesized in most commercial varieties of tobacco (*Nicotiana tabacum*) (Wink and Roberts 1998). In nature, tobacco plants produce basal nicotine levels in the absence of herbivory, and these levels dramatically increase in response to herbivory (Baldwin 1989). Although nicotine is mainly accumulated in the leaf vacuoles and functions as a defensive toxin, the biosynthesis of nicotine occurs in root tissues (Hashimoto and Yamada 2003). For this reason, a tobacco plant requires a variety of nicotine translocation processes from the site of synthesis to the site of accumulation via the long-distance transport.

Root-to-shoot translocation of tobacco alkaloids was first clearly demonstrated in the interspecific grafting experiments between tomato (*Solanum lycopersicum*) and tobacco (Dawson 1942). Analysis of xylem sap taken from the stem, where nicotine was detected as high as 1 mM, indicated that nicotine is synthesized in the roots and transported to the shoots via the xylem stream (Baldwin 1989). In the leaf, nicotine is accumulated in the vacuoles of mesophyll cells, after the movement upward with water (Hashimoto and Yamada 2003). Thus, the translocation of tobacco alkaloids may involve three transport events; export from the synthesizing-cells into the root apoplastic space, uptake into the xylem in the root, and unloading into leaf cells from the xylem.

Biosynthesis of nicotine and related pyridine alkaloids has been studied primarily in *N. tabacum* (Shoji and Hashimoto 2011a). The pyrrolidine ring of nicotine is synthesized from a diamine putrescine by consecutive actions of putrescine *N*-methyltransferase (PMT;

Hibi et al. 1994) and *N*-methylputrescine oxidase (MPO; Heim et al. 2007; Katoh et al. 2007), whereas its pyridine ring is derived from nicotinic acid by way of the de novo biosynthesis pathway of NAD (Katoh et al. 2006), which involves quinolinate phosphoribosyltransferase (QPT; Sinclair et al. 2000). The exact condensation reactions of the two ring systems to produce nicotine are not fully elucidated but require two different types of oxidoreductases, A622 (Shoji et al. 2002; DeBoer et al. 2009; Kajikawa et al. 2009) and BBL (Kajikawa et al. 2011). The tobacco genes encoding these biosynthetic enzymes are coordinately regulated by the jasmonate-signaling cascade (Shoji et al. 2008) and by the *NIC2*-locus transcription factors of an Ethylene Response Factor subfamily (Shoji et al. 2010). These structural genes for nicotine biosynthesis are expressed in the young root tissue, particularly in outer cortex cells, while no substantial expression was observed in aerial parts of tobacco plants (Shoji and Hashimoto 2011a). Together with abundant alkaloid formation in tobacco root cultures (e.g. Solt 1957), the root organ is the site of nicotine biosynthesis in tobacco.

Although genes encoding nicotine biosynthetic enzymes have been characterized extensively, few tobacco alkaloid transporters have been identified. Recently, three nicotine transporters have been reported in tobacco. *N. tabacum* jasmonate-inducible alkaloid transporter 1 (NtJAT1) (Morita et al. 2009) and multidrug and toxic compound extrusion transporters (NtMATE1/2) (Shoji et al. 2009) are tonoplast-localized multisubstrate proton antiporters that take up nicotine into the vacuole from the cytoplasm in tobacco leaf and root cells, respectively. The plasma membrane-localized purine uptake permease-like transporter NUP1 takes up nicotine into tobacco root cells (Hildreth et al. 2011). However, the long-distance translocation of nicotine through the xylem likely requires other transporters or different transport mechanisms, although these three tobacco transporters may be capable of transporting nicotine between different cellular compartments

The Solanaceae is a large angiosperm family that includes many agronomically and

medicinally important crop plants. *Nicotiana* is the sixth largest genus in the Solanaceae and comprises 76 species that are subdivided into 13 sections (Knapp et al. 2004). *Nicotiana* section Alatae (Figure 1) contains eight species with two chromosome numbers, n = 9 and n = 10 (Lee et al. 2008). *N. alata* and *N. langsdorffii* are sister species (n = 9) within the section Alatae (Figure 2). These species have contrasting alkaloid concentrations and profile in the root and leaf. Previous studies revealed that while *N. langsdorffii* accumulates substantial amounts of nicotine in the leaf, tobacco alkaloids are absent or barely detectable in the leaf of *N. alata* (Saitoh et al. 1985; Sisson and Severson 1990; Sinclair et al. 2004). Such distinct alkaloid profiles in the leaf of two closely related *Nicotiana* species offer an excellent opportunity to compare their ability to translocate alkaloids and to investigate the genetic basis of this trait. Both species are predominantly self-incompatible and no hybrids have been observed in natural populations. However, they can be intercrossed with each other in the greenhouse (Lee et al. 2008; Lim et al. 2006). Previously, plant physiologists hybridized *N. alata* and *N.langsdorffii*, but only analyzed the inheritance of morphological traits (East 1916).

In Chapter I, I report an analysis of the translocation phenotype of *N. langsdorffii*, *N. alata* and their interspecific hybrids, and discuss the inheritance patterns of the alkaloid translocation phenotype.

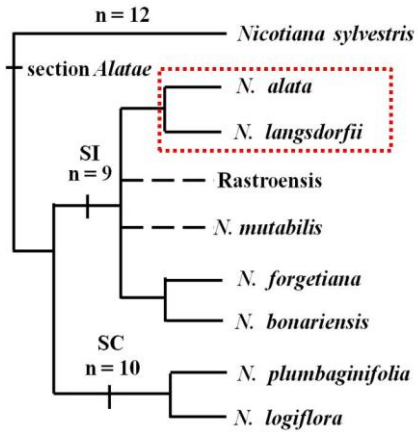

Figure 1. Phylogenetic relationship of *Nicotiana* section Alatae. *N. alata* and *N. langsdorffii* are sister species within section Alatae. The SI (self-incompatible) n = 9 clade of section Alatae is comprised of eight species, but the phylogenies have not been resolved for *N. mutabilis* and putative species 'Rastroensis' (dashed lines). The SC (self-compatible) n = 10 clade consists of *N. longiflora* and *N. plumbaginifolia*. From Lee et al. 2008.

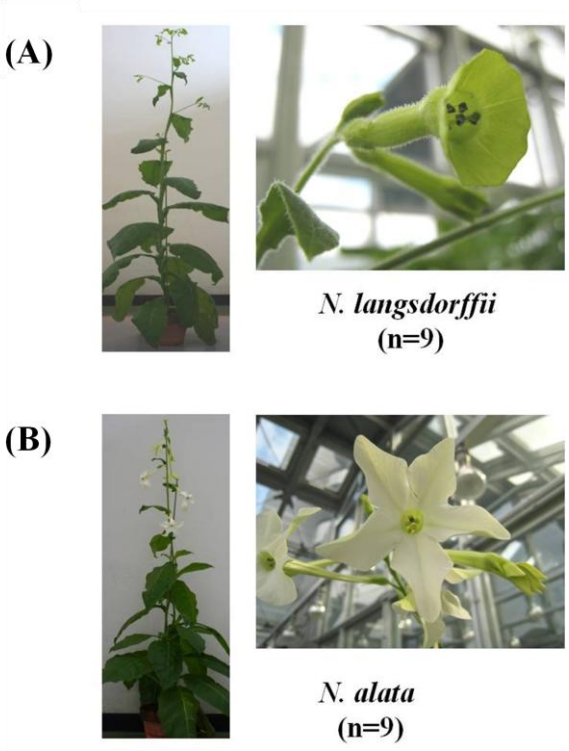

Figure 2. Two closely related *Nicotiana* species, *N. langsdorffii* and *N. alata*. (A) A mature *N. langsdorffii* plant at the flowering stage. (B) A mature *N. alata* plant at the flowering stage. n: chromosome number

Materials and Methods

Plant materials

Seeds of *Nicotianan alata* Link & Otto and *N. langsdorffii* Weinmann were obtained from Leaf Tobacco Research Center, Japan Tobacco Inc. (Oyama, Japan). Since *N. alata* plants showed strong self-incompatibility, they were maintained by out-crossing. Plants were grown under greenhouse conditions with a 16-h-light/8-h-dark cycle, in a compound soil mixture, which consisted of one part of Supermix-A (Sakata Seed Co., Japan), one part of Akadama tsuchi (Sakata Seed Co., Japan), two parts of vermiculite and 1/8 part of an Osmocote slow release fertilizer (http://www.hyponex.co.jp). The temperatures in the greenhouse were maintained at 28 ℃ during the day and at 18 ℃ during the night. To create interspecific F_1 hybrids, the pollen of *N. alata* was crossed to the pistil of *N. langsdorffii*.

Alkaloid analysis

When harvesting tobacco roots from soil-grown plants, I carefully removed attached soil from the roots so that fine lateral roots and root tips were not damaged. Harvested leaves and roots (50 mg dry weight) were lypophilized, homogenized and soaked in 4 ml of 0.1N H_2SO_4. The homogenates were sonicated for 60 min and centrifuged for 15 min at 2,000 rpm. One milliliter of the supernatant or the culture medium (in cases when alkaloids in the liquid culture were analyzed) was mixed with 0.1 mL of concentrated NH_4OH, and the mixture (1 mL) was applied to an Extrelut-1 column (Merck). Chloroform (6 mL) was passed through the column, the chloroform eluent was evaporated to dryness at 37℃, and the dry residues were dissolved in ethanol containing 0.1% (v/v) dodecane as an internal standard. The quantitative analysis of tabacco alkaloids was carried out by gas-liquid chromatography (GC-2010 Shimadzu) with a Rtx-5 Amine capillary column (Restek). The column temperature

was as a thermal gradient: 100°C for 3 min; 25°C min^{-1} to 150°C; 2°C min^{-1} to 170°C and 30°C min^{-1} to 300°C.

RNA gel blot analysis

Total RNAs were prepared by using RNeasy kit (Qiagen) from leaves and roots of 6 week-old plants grown on soil in greenhouse. Ten micrograms of total RNA was loaded onto each lane and separated by electrophoresis on 1.2% formaldehyde gel. Equal loading for each lane was confirmed by staining the gels with ethidium bromide before blotting onto a Hybond N+ nylon membrane (Amersham). RNA gel blots were probed with 32P-labelled full-length coding sequences of tobacco *PMT* (Hibi et al. 1994), *MPO1* (Katoh et al. 2007), *QPT2* (Shoji and Hashimoto 2011b), *A622* (Hibi et al. 1994), *NtMATE1*(Shoji et al. 2009), and *CYP82E4* (Siminszky et al. 2005). Labeling was done with Bcabest labeling kit (Takara). CYP82E4 cDNA was cloned by RT-PCR from RNA of tobacco leaves treated with ethylene gas for 24 h as described (Shoji et al. 2000).

PCR genotyping

Genomic DNA for genotyping was extracted from leaves using the PureLink Plant Total DNA Purification Kit (Invitrogen) by following the manufacturer's instructions. Primers were designed to differentiate the *WIN4* alleles (Kodama and Sano 2007) and the *MATE* alleles (Shoji et al. 2009) of *N. langsdorffii* and *N. alata***.** The forward and reverse primers, respectively, were 5'-GTATCGGCGAATCATCGGAACCTG-3' and 5'-GAGAACCTCTA GCATTGAATCTATCCC-3', for the *WIN4* locus, and 5'-AGAAGTATCTGAGTTGTGT CC-3' and 5'-CGTTTAAGACCAAATTGGGTAAGC-3', for the *MATE* locus. The PCR amplification conditions were: 32 cycles of 30 sec at 94°C, 30 sec at 55°C, 2 min at 72°C, with an initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 7

min. Amplified PCR products were separated on 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

Grafting experiments

Reciprocal grafting was made on 21-day-old seedlings of *N. langsdorffii* and *N. alata*. Grafted plants (approximately four grafts for each combination) were kept under high humidity and dark conditions for two weeks to promote graft union, after which the vigorously growing grafted plants were grown under the normal greenhouse conditions.

Xylem sap collection

Before collection of xylem sap, 1.5-mL Eppendorff tubes were stuffed with silica (Quartz) wool up to half of the tube volume, and a small hole was made at the bottom of each tube. Stems of four-week-old plants were cut with a sharp razor blade about 5 cm above the soil level, and the cut surface was then rinsed with distilled water and wiped dry. Xylem sap was collected from the cut end for two hours into the collection tube, and the sap collection was repeated one more time from the same plant. The sap adsorbed to the silica wool was recovered in a new 1.5-mL tube by brief centrifugation. All sap samples were frozen immediately after sampling, and stored at -20°C until analyzed. Xylem sap collected as described above was used for determination of tobacco alkaloid content.

Hairy root culture for production of tobacco alkaloid

Leaves of 6-week-old plants (*N. langsdorffii* and *N. alata*) were cut into 1×1 cm discs and dipped in the suspension of *Agrobacterium rhizogenes* strain 15834 for 5 min, dried on sterile filter papers, and incubated in the dark at 27°C on the Murashige and Skoog's solid medium. After two days of co-culture, the infected leaves were transferred to the solid

medium containing 250 mg L^{-1} cefotaxime to eliminate the bacteria. The adventitious roots appeared from the leaf discs two weeks after the infection, and the induced hairy roots were subcultured on the same medium every two weeks at 27°C in the dark. After two subcultures, the root clones with no apparent bacterial contamination were transferred to the Gamborg's B5 liquid medium without antibiotics, and cultured on a rotary shaker at 80 rpm, 27°C in the dark. Hairy roots were transferred to a fresh medium every two weeks. One month later, the hairy root cultures were harvested for analysis of tobacco alkaloid contents. To determine alkaloids in the culture medium, the medium was directly applied into an Extrelut-1 column and then processed according to the above alkaloid analysis.

Results

Hybrid verification

I crossed *N. alata* (as a male parent) to *N. langsdorffii* (as a female parent) to generate F_1 hybrids since hybridization between these two species is reported to be more efficient in this combination (Lee et al. 2008). PCR-based markers that distinguish between *N. alata* and *N. langsdorffii*, the *WIN4* primers were developed at the *WIN4* locus (Kodama and Sano 2007) and the *MATE* primers, which corresponded to the tobacco *MATE1/2* genes (Shoji et al. 2009), were used to verify the F_1 interspecific hybrid status. The *WIN4* primers amplified a 0.7-kb genomic fragment from *N. langsdorffii*, a 1.1-kb fragment from *N. alata*, and both fragments from the F_1 hybrids, whereas the *MATE* primers generated a 1.1-kb fragment from *N. langsdorffii*, a 0.7-kb fragment from *N. alata*, and both fragments from the F_1 hybrids (Figure 3A). Thus, these results confirmed that the progeny were indeed F_1 interspecific hybrids.

Morphological comparison of the parent species and their F_1 hybrid offspring

N. alata and *N. langsdorffii* do not differ as strikingly in vegetative characters as they do in floral characters. Mature *N. langsdorffii* plants are approximately 1.5 m tall and produce many axillary stems, whereas *N. alata* plants at the flowering stage are smaller (approximately 1 m tall), have many fewer axillary stems, and show wrinkled margins of pointed leaves. The F_1 hybrids (Figure 3B) were morphologically intermediate between the parents in many characters, with a few axillary stems and oval leaves with weakly wrinkled margins. The parental species show distinct flower morphology: the *N. langsdorffii* flower is composed of five petals completely fused to form a disk of about 2 cm in diameter, and a limegreen tube, while the much larger *N. alata* flower (about 10 cm long) has five petals

partially fused at their bases, and is chalky white (Figure 3C). Moreover, *N. langsdorffii* has bright blue pollen, while that of *N. alata* is ivory-colored. The hybrid flower is lime green with dark blue pollen, and shows a morphology that is intermediate between those of the two parents.

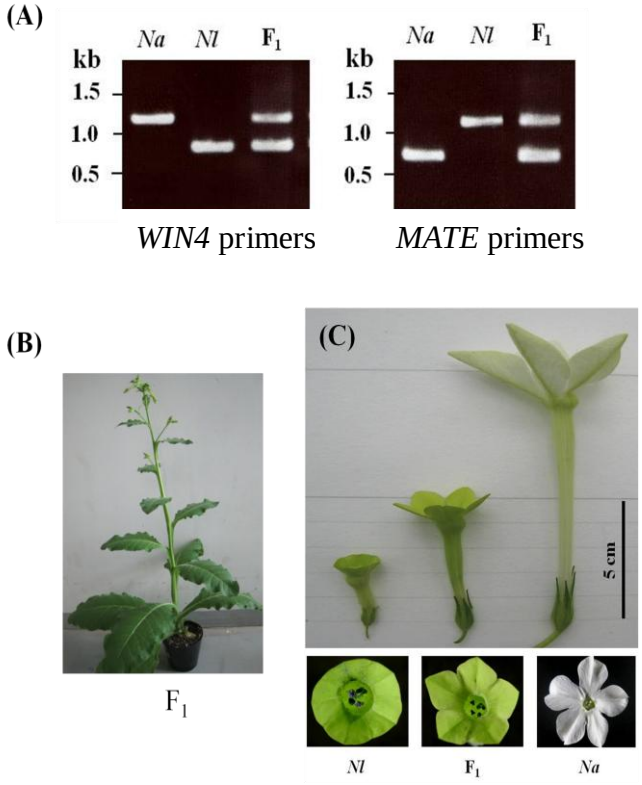

Figure 3. The F_1 interspecific hybrid of *N. langsdorffii* (*Nl*) and *N. alata* (*Na*). (A) PCR genotyping of *N. alata*, *N. langsdorffii*, and their F_1 hybrid progeny at the polymorphic *WIN4* and *MATE* loci. The F_1 plant contains *WIN4* and *MATE* alleles derived from both *N. alata* and *N. langsdorffii*. (B) A mature F_1 hybrid plant at the flowering stage. (C) Comparison of floral phenotype in *N. langsdorffii*, *N. alata* and F_1 hybrid. The F_1 flower shows phenotypes of intermediate between both parents.

Expression of nicotine biosynthesis genes (this subsection was done by Dr. Tsubasa Shoji)

To examine the site of alkaloid biosynthesis, expression of representative structural genes involved in biosynthesis (*PMT*, *MPO*, *QPT*, *A622)*, metabolism *(CYP82E)*, or transport of nicotine (*MATE1/2)*, in the root and the leaf of *N. langsdorffii* and *N. alata*, were analyzed by RNA gel blot analysis (Figure 4). Expression of *PMT*, *MPO*, *QPT*, *A622*, and *MATE1/2* was observed in the root, but not in the leaf, of *N. langsdorffii* and *N. alata*, while *CYP82E* was preferentially expressed in the leaf and the root of *N. alata* but not of *N. langsdorffii*. Thus, nicotine and probably other pyridine alkaloids are synthesized in the root of these wild *Nicotiana* species, and the conversion of nicotine to nornicotine occurs both in the leaf and the root of *N. alata*, which is consistent with the well established sites of nicotine biosynthesis in *N. tabacum* (Shoji and Hashimoto 2011a). Although nicotine conversion occurs predominantly in the leaf of *N. tabacum* (Chakrabarti et al. 2008), the conversion appears to be active both in the root and the leaf of *N. alata*.

Distribution of tobacco alkaloids in plants

I next measured tobacco alkaloids in the leaf and the root of *N. langsdorffii*, *N. alata*, and their F_1 hybrids. When 10-week-old mature plants were analyzed, nicotine was the predominant alkaloid in the leaf and the root of *N. langsdorffii*, with much smaller amounts of anatabine found in the root, whereas considerable amounts of pyridine alkaloids were found only in the root, but not in the leaf, of *N. alata* (Figure 5). In the root of *N. alata*, nicotine was the major alkaloid, followed by nornicotine and anatabine. Restricted presence of nornicotine in *N. alata* is consistent with expression of the nicotine *N*-demethylase gene (*CYP82E*) in *N. alata*, but not in *N. langsdorffii* (Figure 4). The F_1 hybrid plants accumulated pyridine alkaloids (which consisted mostly of nicotine, with smaller amounts of anatabine and nornicotine) in the root; no substantial amounts of alkaloids were found in the leaf. These

results indicate that the alkaloids synthesized in the root are readily transported to the leaf in *N. langsdorffii*, but this inter-organ translocation does not occur in *N. alata* and the F_1 hybrid.

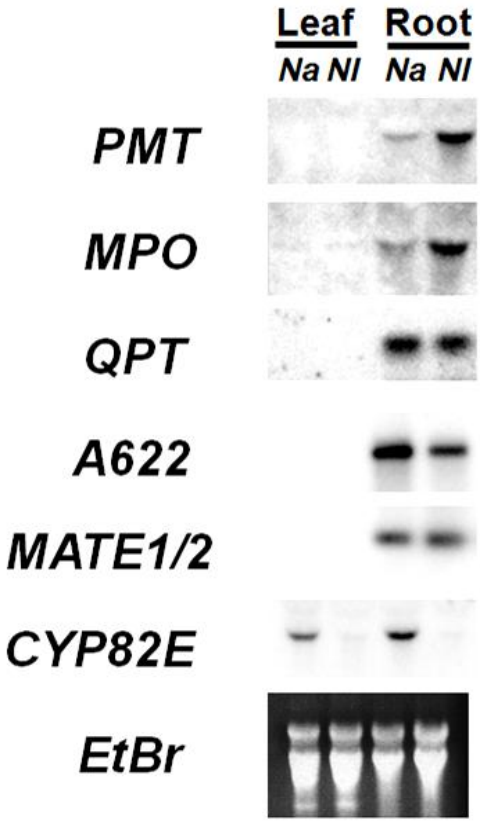

Figure 4. RNA gel blot analysis of the genes involved in biosynthesis and transport of pyridine alkaloids. Gene expression in the leaf and the root of *N. langsdorffii* (*Nl*) and *N. alata* (*Na*) was analyzed. PMT (Hibi et al. 1994), MPO (Katoh et al. 2007), QPT (Sinclair et al. 2000), and A622 (Kajikawa et al. 2009) are involved in biosynthesis of nicotine, whereas CYP82E metabolizes nicotine to nornicotine (Siminszky et al. 2005). MATE1/2 transporters facilitate uptake of nicotine into vacuoles in tobacco roots (Shoji et al. 2009). The staining with ethidium bromide shows equal loading of total RNA on each lane.

* This figure is based on the results obtained by Dr. Tsubasa Shoji.

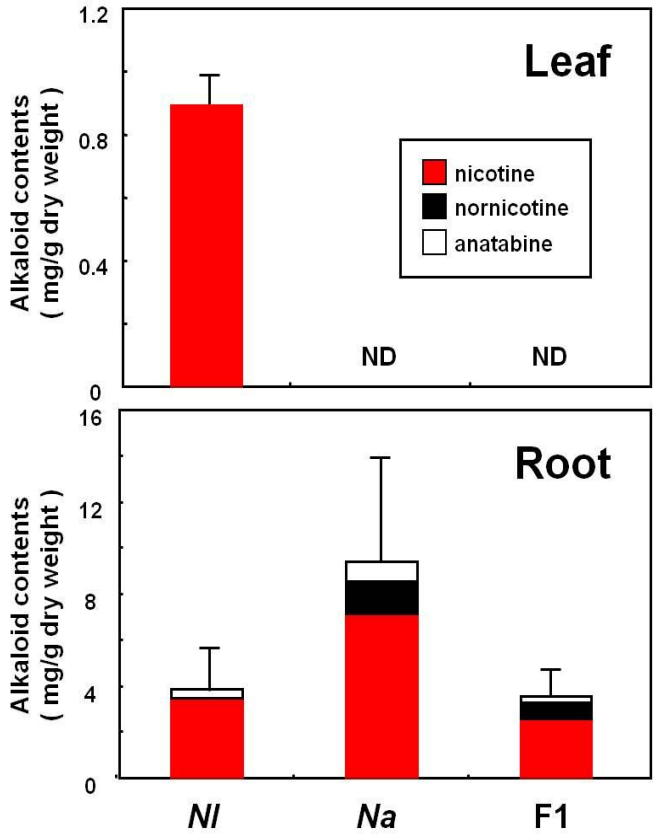

Figure 5. Alkaloid content in 10-week-old plants of *N. langsdorffii (Nl), N. alata (Na)* and their F_1 hybrid. Nicotine, nornicotine and anatabine in the leaf (upper) and root (lower) is shown in the indicated colors. Error bars indicate the SD for four biological replicates. ND: not detected.

The root of *N. alata* is deficient in alkaloid export

To evaluate contribution of the root organ and the aerial parts on the long-distance translocation of alkaloids, I performed interspecific grafting experiments in which *N. langsdorffii* scions were grafted onto *N. alata* stocks and vice versa (Figure 6A). As controls, homologous grafting combinations (*N. langsdorffii* scions on *N. langsdorffii* stocks , and *N. alata* scions on *N. alata* stocks) were prepared, which served as controls to prove that upward movement of tobacco alkaloids occurred quite normally during the grafting procedure. One month after grafting, alkaloids were analyzed in the leaf and the root of grafted plants. The

control *N. langsdorffii* plants accumulated mostly nicotine in the leaf and the root, while the control *N. alata* plants contained nicotine as a major alkaloid, followed by nornicotine and anatabine, in the root but did not accumulate substantial amount of tobacco alkaloids in the leaf. These results are consistent with those obtained in the non-grafted plants (Figure 6B). Interspecifically grafted plants with *N. alata* scion and *N. langsdorffii* stock accumulated mostly nicotine both in the root and the leaf. In contrast, grafted plants with the reverse combination (*N. langsdorffii* scion on *N. alata* stock) accumulated tobacco alkaloids in the root similar in contents and compositions to *N. alata* plants, but did not contain substantial amounts of alkaloids in the leaf. These results indicated that no alkaloid movement occurs from *N. alata* rootstock, but alkaloids can flow upward from *N. langsdorffii* rootstock.

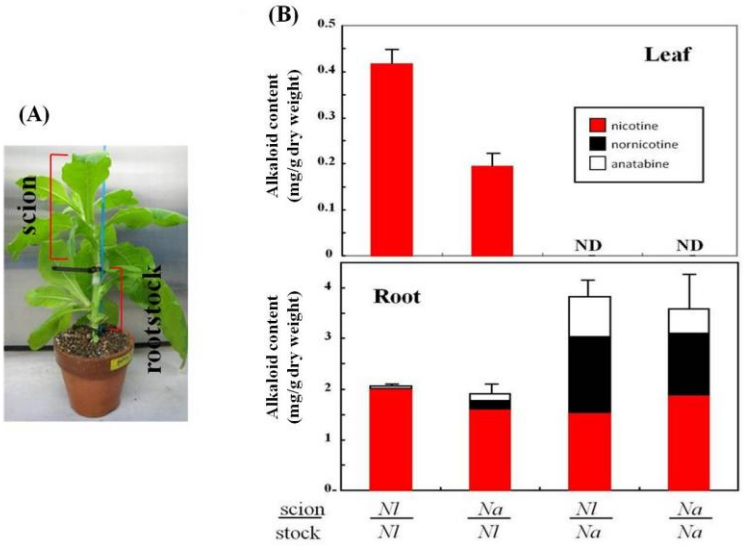

Figure 6. Tobacco alkaloid translocation in grafted plants. (A) A grafted plant. (B) Alkaloid content in interspecifically grafted plants. *N. langsdorffii (Nl)* and *N. alata (Na)* were reciprocally grafted, and alkaloid content in the leaf and root was analyzed one month after grafting. The plant species used for scion and stock are indicated. Error bars indicate the SD for four biological replicates. ND: not detected.

Because nicotine is believed to be transported from the root to aerial parts via the xylem (Wink and Roberts 1998), I measured the contents of tobacco alkaloids in the xylem sap of 4-week-old plants (Figure 7A). Nicotine was present at the concentration of 4.3 μM in the xylem sap of *N. langsdorffii*, but was not detectable in the xylem saps of *N. alata* and the F_1 hybrid (Figure 7B). These results suggest that nicotine is not mobilized from the alkaloid synthesizing cells into the xylem in the root of *N. alata* and the F_1 hybrid.

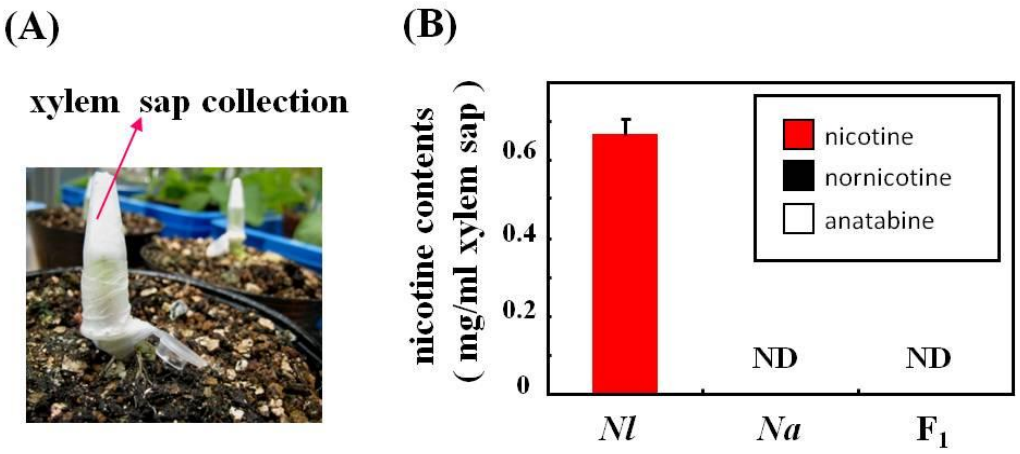

Figure 7. Tobacco alkaloids in xylem sap. (A) Collection of xylem sap. (B) Alkaloid content in the xylem sap of *N. langsdorffii* (*Nl*), *N. alata (Na),* and their F_1 hybrid. Error bars indicate the SD for four biological replicates. ND: not detected.

To examine the ability of root tissues to export alkaloids out of the synthesizing cells, I established hairy root cultures from *N. langsdorffii* and *N. alata* (Figure 8A). *N. langsdorffii* hairy roots synthesized nicotine, while *N. alata* hairy roots produced primarily nornicotine, with smaller amounts of nicotine and anatabine (Figure 8B). In my root cultures, *N. alata* roots produced larger amounts of tobacco alkaloids than *N. langsdorffii* roots. Despite their

higher capacity to synthesize alkaloids, the culture medium of *N. alata* hairy roots contained very low amounts of tobacco alkaloids, compared to the culture medium of *N. langsdorffii* hairy roots. These results indicate that alkaloids produced in the root tissues of *N. alata* are exported from the synthesizing-cells, and are consistent with the absence of tobacco alkaloids in the xylem sap of *N. alata* plants.

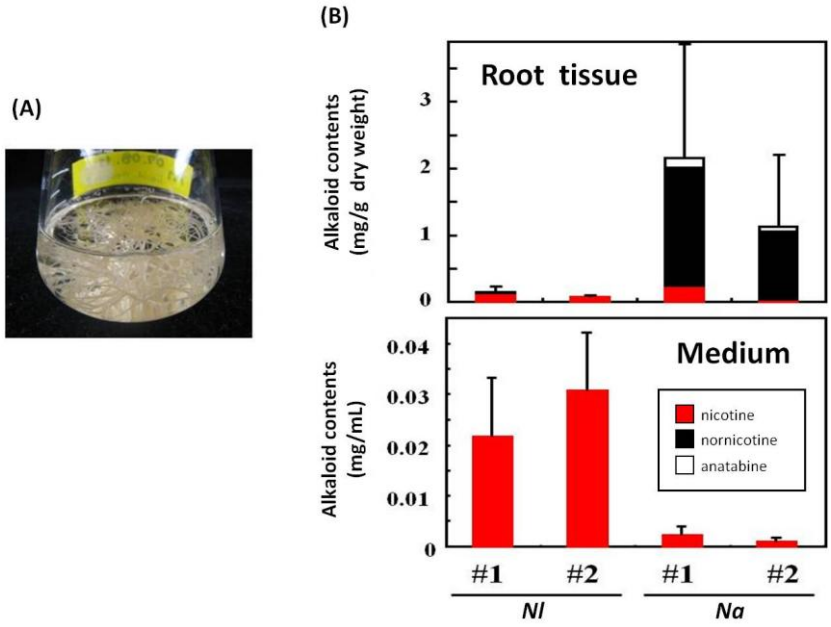

Figure 8. Secretion of alkaloids. (A) Hairy root culture in liquid culture medium. (B) Alkaloid content in the root cells and culture medium of root cultures of *N. langsdorffii (Nl) and N. alata (Na).* Two independent root culture lines (#1 and #2) were analyzed for both species. Error bars indicate the SD for four biological replicates

Genetic analysis of alkaloid translocation

To examine genetic loci involved in alkaloid translocation, I first analyzed alkaloid content in the leaf of 109 individual F_2 plants derived from the self-pollination of an F_1 hybrid

plant between *N. langsdorffii* and *N. alata* (Figure 9A). Among these plants, 46 plants (42.2 %) accumulated nicotine or nornicotine in the leaf at appreciable amounts, but the alkaloid contents varied considerably from 3.1 mg g dry weight^{-1} to 0.1 mg g dry weight^{-1}. F_2 plants with alkaloids at less than 0.5 mg g dry weight^{-1} constituted 71.7 % of the alkaloid accumulating plants (n = 33). The data is suggestive of complex genetic arrangement, probably involving two or more semi-dominant loci. Nornicotine predominated in 26 plants while the remaining 20 plants accumulated almost exclusively nicotine. The *CYP82E* genes derived from *N. alata* may be responsible for the nornicotine phenotype. I next extended my analyses to the backcrossed progenies. Successful backcrosses were confirmed by genotyping individual plants with the *WIN4* markers (Figure 10). When an F_1 hybrid was backcrossed to *N. langsdorffii*, 12 plants (32.4 %) contained considerable amounts of alkaloids (10 nicotine-type plants, and 2 nornicotine-type plants) in the leaf among 37 plants analyzed, suggesting a proportion of alleles from the *N. langsdorffii* translocating parent genome was recover (Figure 9B). On the other hand, all the plants (n = 38) produced by backcrossing an F_1 hybrid to *N. alata* did not contain detectable amounts of alkaloids in the leaf (Figure 9C). These backcrossing experiments support that tobacco alkaloids synthesized in the root are retained in the organ by the action of a few (semi)dominant loci in *N. alata*.

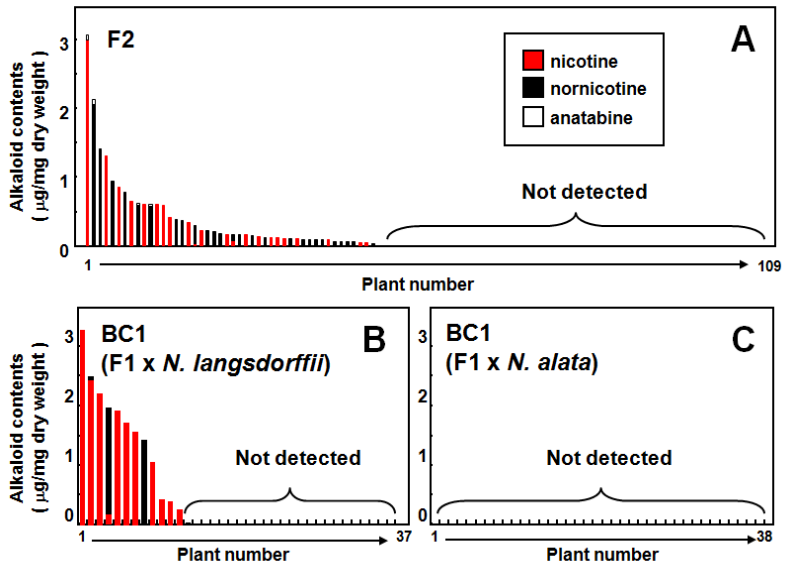

Figure 9. Alkaloid contents in the leaf of progeny plants derived from the F_1 hybrid between *N. langsdorffii* and *N. alata*. Analyzed plants were numbered from 1 to the indicated number. (A) F_2 plants derived from self-pollination of the F_1 plant. (B) Backcrossed progeny produced by crossing F_1 (male) to *N. langsdorffii* (female). (C) Backcrossed progeny produced by crossing F_1 (male) to *N. alata* (female).

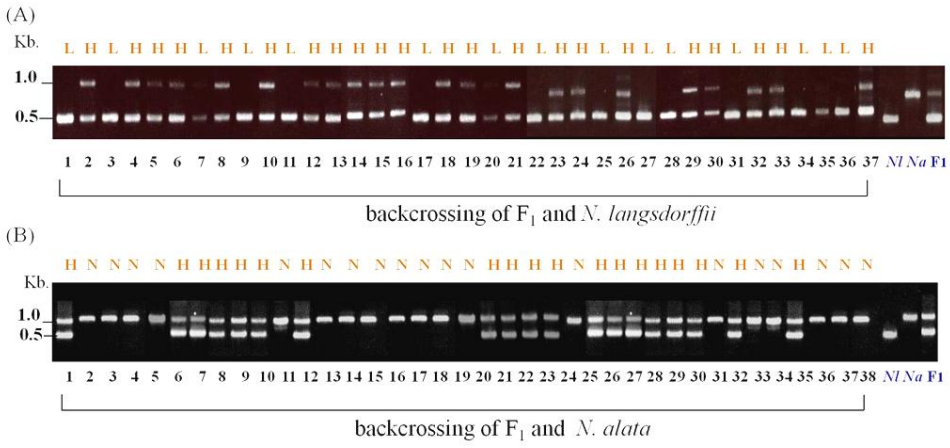

Figure 10. Amplification pattern generated by the *Win 4* primers for genotyping of backcross populations. (A) PCR genotyping of the backcrossing of F_1 and *N. alata (Na)*. N: *N. alata* genotype, H: heterozygous genotype. (B) PCR genotyping of the backcrossing of F_1 and *N. langsdorffii (Nl)*. L: *N. langsdorffii* genotype, H: heterozygous genotype.

Discussion

Pyridine alkaloids (nicotine, anatabine, anabasine, and nornicotine) generally accumulate at considerable levels in the leaves of *Nicotiana* plants. A notable exception is the ornamental tobacco *N. alata*, in which pyridine alkaloids are absent or present at very low levels in leaves (Saitoh et al. 1985; Sisson and Severson 1990) even after wounding to the foliage (Sinclair et al. 2004). Roots of *N. alata* plants do contain appreciable levels of nicotine, anatabine, and nornicotine (Saitoh et al. 1985, Friesen et al., 1992, Sinclair et al. 2004). My alkaloid analysis of *N. alata* plants is generally consistent with these previous reports.

The root organ is the major site of alkaloid biosynthesis in various *Nicotiana* species (Shoji and Hashimoto 2011a), with an exception of the tree tobacco *N. glauca*, which appears to synthesize at least part of anabasine in leaves (Sinclair et al. 2004). Presence of high levels of tobacco alkaloids in *Nicotiana* leaves appears to result from efficient translocation of alkaloids from roots to aerial parts via the xylem (Wink and Roberts 1998). Presence of appreciable amounts of tobacco alkaloids in the xylem sap is consistent with the proposed apoplastic transport through the xylem network (Baldwin 1989; Figure 7B). Expression of alkaloid biosynthesis genes in the root (Figure 4) and formation of alkaloids in the root cultures (Figure 8B; Friesen et al.1992; Sinclair et al., 2004) demonstrate that the root of *N. alata* is capable of synthesizing pyridine alkaloids. Thus, in *N. alata*, the absence of alkaloids in leaves is likely attributable to an inefficient long-range alkaloid translocation.

Several bacterial species have the ability to use nicotine as its sole carbon and energy source by oxidatively degrading the alkaloid through a series of enzymatic steps (Brandsch 2006). Specific degradation of alkaloids in the *N. alata* leaves might account for the absence of pyridine alkaloids in that organ, although enzymatic degradation of tobacco alkaloids has not been reported in *Nicotiana* plants. The *N. alata* scion grafted onto the *N. langsdorffii* stock accumulates high levels of nicotine in the leaf (Figure 6B**)**, providing evidence that the

tobacco alkaloids once transported to the *N. alata* leaves are scarcely degraded. I conclude that *N. alata* is not capable of mobilizing tobacco alkaloids from the root to the leaf. The root-to-leaf translocation involves export of alkaloids from the synthesizing cells in the root, alkaloid uptake into the xylem, and alkaloid unloading into leaf cells from the xylem. I did not detect tobacco alkaloids in the xylem sap of *N. alata* plants (Figure 7B), suggesting that a process prior to the xylem loading in the root does not occur in *N. alata*. Low secretion of alkaloids into the culture medium of cultured *N. alata* hairy roots (Figure. 8B) is consistent with this.

Alkaloid analysis of the F_1 hybrid plants between *N. alata* and *N. langsdorffii*, their selfed F_2 progeny plants, and sibling plants obtained from backcrossing the F_1 plants to either parent (Figure 5 and Figure 9) clearly showed that the non-translocation trait is genetically dominant over the translocation trait. The segregation data of leaf alkaloids indicate that more than one dominant locus is involved but further studies are necessary to characterize the genetics of alkaloid translocation. What might be the molecular identity of the dominantly acting non-translocation phenotype in *N. alata*? I speculate that a structural or biochemical blockage which impedes alkaloid export from synthesizing cells or alkaloid uploading into the xylem has evolved in the root of *N. alata*. High-resolution anatomic studies of the root tissues might provide clues to understand the mechanisms of alkaloid retention in the *N. alata* roots. Depletion of tobacco alkaloids in the aerial parts of *N. alata* may necessitate this tobacco species to develop or strengthen alternative defense strategies against herbivores, such as production of proteinase inhibitors and emission of volatile chemicals that attract predatory bugs (Baldwin 2001). In addition, accumulating toxic alkaloids in the root system may contribute more enhanced protection of the underground organ against herbivores that live in the soil, such as nematodes.

Although this study focused on alkaloid distribution in leaves and roots, the genetic

materials generated in this work may be used to study other aspects of alkaloid biology. Nornicotine is found in *N. alata* but is absent in *N. langsdorffii*. In *N. tabacum*, several differentially expressed *CYP82E* genes encoding nicotine *N*-demethylase contribute conversion of nicotine to nornicotine in different tissues (Siminszky et al. 2005, Gavilano and Siminszky 2007, Lewis et al. 2010). Molecular genetic analysis of the *CYP82E* genes in *N. alata*, *N. langsdorffii*, and their hybrid progenies may provide molecular mechanisms on dysfunctionalization of *N. alata CYP82E* genes and contribution of individual *N. langsdorffii CYP82E* genes for nornicotine formation in wild *Nicotiana* species.

CHAPTER II

Inactivation of two *CYP82E* nicotine *N*-demethylase genes abolishes nornicotine formation in *Nicotiana langsdorffii*

Introduction

The four major alkaloids found in tobacco leaves are nicotine, nornicotine, anabasine and anatabine (Figure 11A). Alkaloid production is induced by plant tissue damage and serves as a defense against herbivore attack. In cultivated tobacco (*Nicotiana tabacum* L.) nicotine is the most abundant alkaloid, typically constituting more than 90% of the total alkaloid pool. However, not all species in the genus *Nicotiana* are characterized by high levels of nicotine in their foliage. Alkaloid composition and concentration vary greatly among and within *Nicotiana* species. In *N. glauca*, for example, anabasine is the most abundant alkaloid, while foliage of *N. tomentosiformis* contains predominantly nornicotine (Saitoh et al. 1985; Sisson and Severson 1990). Nornicotine was also reported as the major alkaloid in *N. sylvestris* (Goodspeed 1954; Gerstel 1961). Although the primary alkaloid is nicotine in the green leaves of *N. sylvestris*, almost the entire nicotine content of the leaf is metabolized into nornicotine during senescence (Chakrabarti et al. 2008). In *N. tabacum*, the content of the minor alkaloid nornicotine usually represents less than 5% of the total pyridine alkaloid pool (Saitoh et al. 1985). Nevertheless, nornicotine production and accumulation in cultivated tobacco are undesirable because nornicotine serves as the main precursor to tobacco-specific nitrosamine *N*′-nitrosonornicotine (NNN), a potent carcinogen. NNN is naturally generated via the nitrosation of nornicotine during the curing and processing of harvested tobacco leaves. Leaves containing high levels of nornicotine tend to contain high levels of NNN (Chakrabarti et al. 2008). High levels of nornicotine also adversely affect tobacco quality by producing

undesirable flavor and smoking characteristics, such as an alkaline taste and mousy aroma in tobacco products. Thus, in cultivated tobacco, nicotine-accumulating tobacco is preferentially selected over nornicotine-accumulating (Roberts 1988).

Nicotine is synthesized in tobacco roots by several enzymatic steps (Shoji and Hashimoto 2011a) and then translocated through the xylem to aerial parts of the plant (Dawson 1942; Hashimoto and Yamada 2003). In some *Nicotiana* plants, leaf nicotine is further converted to nornicotine during leaf senescence and curing (Wernsman and Matzinger 1968). Both biochemical and chemical evidence have shown that nornicotine is primarily produced via the oxidative *N*-demethylation of nicotine by nicotine *N*-demethylase (NND) (Leete 1983; Bush et al. 2001). The nicotine demethylase gene has recently been isolated, and was found to encode a cytochrome P450 monooxygenase, designated as the *CYP82E* gene subfamily (Figure 11B; Siminszky et al. 2005). Until recently, several nicotine demethylase genes that mediate nornicotine biosynthesis in *Nicotiana* plants have been well characterized. *N. tabacum* contains at least five *CYP82E*-related genes: *CYP82E4*, *CYP82E5*, and *CYP82E10* encode functional nicotine *N*-demethylases (Siminszky et al. 2005; Gavilano and Siminszky 2007; Lewis et al. 2010), whereas *CYP82E2* and *CYP82E3* encode inactive enzymes (Chakrabarti et al. 2008; Gavilano et al. 2007). *N. tabacum* is an allotetraploid, produced by hybridization of diploid *Nicotiana* species closely related to modern *N. sylvestris* and *N. tomentosiformis* (Murad et al. 2002). Molecular characterization of the conversion factor showed that *NtabCYP82E3*, *NtabCYP82E4* and *NtabCYP82E5v2* were donated by the *N. tomentosiformis* parent. *NtomCYP82E3* expressed high levels of NND activity, but in tobacco the orthologous *NtabCYP82E3* gene is inactivated by the W330C knock-out mutation (Siminszky et al. 2005; Gavilano and Siminszky 2007; Gavilano et al. 2007; Chakrabarti et al. 2007). Moreover, *NtabCYP82E2* and *NtabCYP82E10* were found to originate from the *N. sylvestris* genome. Unlike *NtabCYP82E2*, the *N. sylvestris* ortholog *NsylCYP82E2* exhibited

NND activity in senescing leaves. The *NtabCYP82E2* gene is inactivated by two knock-out mutations (Chakrabarti et al. 2007; Lewis et al. 2010). These findings indicated that the inactivation of *NtabCYP82E2* and *NtabCYP82E3* genes played a major role in the evolution of the alkaloid composition of modern tobacco.

The results presented in Chapter I demonstrated that the ability to translocate tobacco alkaloids and alkaloid composition differs between *N. langsdorffii* and *N. alata*. Alkaloid profile showed that *N. langsdorffii* accumulates substantial amounts of nicotine and anatabine in the root and the leaf, but does not contain nornicotine anywhere, whereas nicotine, anatabine, and nornicotine are produced exclusively in the root of *N. alata*. Interestingly, nicotine-to-nornicotine conversion occurs in *N. alata* root, whereas nornicotine is usually reported to be accumulated in the leaf in *Nicotiana* species (Siminszky et al. 2005; Gavilano and Siminszky 2007; Chakrabarti et al. 2008; Gavilano et al. 2007). There is thus a distinct difference in nicotine conversion between *N. langsdorffii* and *N. alata*, indicating a non-converting phenotype in *N. langsdorffii* and a nicotine-converting phenotype in *N. alata*.

In Chapter II, therefore, I extended my investigation to the molecular mechanism associated with inactivation of nicotine-to-nornicotine conversion in *N. langsdorffii*, and the inheritance of nicotine-to-nornicotine conversion in wild *Nicotiana* species.

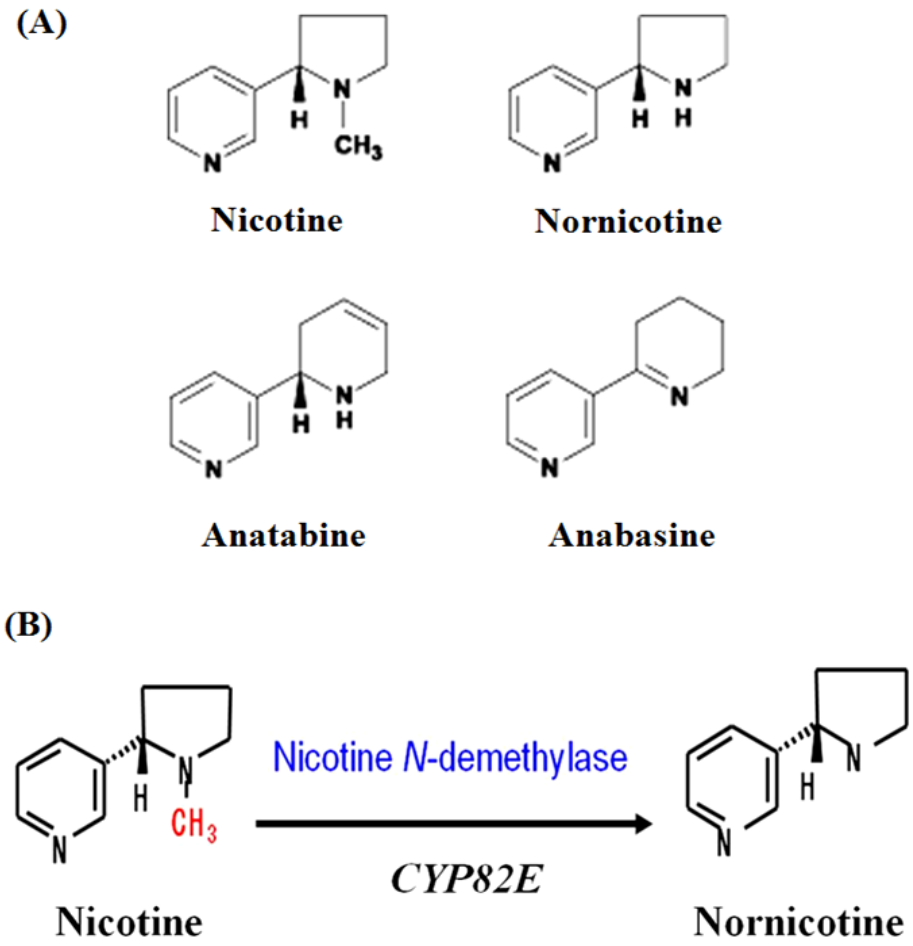

Figure 11. Chemical structures of pyridine alkaloids and conversion of nicotine to nornicotine. (A) Structures of nicotine and related pyridine alkaloids identified in *Nicotiana* species. (B) Biosynthetic pathway of nicotine to nornicotine conversion. Nicotine is enzymatically converted to nornicotine by nicotine *N*- demethylase (NND).

Materials and Methods

Nornicotine analysis

Roots were harvested, processed, and analyzed for tobacco alkaloids by gas-liquid chromatography, as described previously in Chapter I. In the study, percent nicotine conversion was used to express the conversion level of plant samples. The formula used for calculating percent nicotine conversion was

$$\% \text{ nicotine conversion} = 100 \times \text{nornicotine content} / (\text{nicotine content} + \text{nornicotine content})$$

When necessary, plants were grouped into three categories: non converter (no nornicotine detected), low converter (detectable nornicotine but less than 20% conversion), and high converter (exceeding 20% conversion).

Cloning of *CYP82E* cDNAs

Total RNA was extracted from leaves and roots using the Qiagen RNeasy Mini kit according to the manufacturer's instruction (Qiagen), and was used to synthesize first strand cDNA by using SuperScript II Reverse Transcriptase kit (Invitrogen). The cDNA fragments of *CYP82E* were PCR-amplified by the primers which were designed based on the conserved nucleotide sequences of the published *CYP82E* subfamily.

Full-length *CYP82E* cDNA clones were obtained by amplification of 5'- and 3'-ends of the cDNA, according to the protocol described in SMART RACE cDNA Amplification Kit (Clontech). The cycle conditions for the nested-PCR were 94°C for 3 min followed by 30 cycles 94°C for 30 sec, 65°C for 30 sec, 72°C for 3 min, and a final extension at 72°C for 7 min. PCR products were purified, cloned in pGEM-T Easy (Promega), and sequenced by using ABI PRISM 3100 automated DNA sequencer. All the PCR primers used in cloning

CYP82E cDNAs are listed in Table S2.

Cloning of *CYP82E* genomic fragments

Genomic DNA was extracted from leaves using the PureLink Plant Total DNA Purification Kit (Invitrogen) by following the manufacturer's instructions. Genomic fragments containing introns and part of the exons of the *CYP82E* genes were obtained by amplifying genomic DNA with PCR primer sets which were specific to each *CYP82E* gene. 5'- and 3'-regions of *NlanCYP82E1* were obtained by inverse PCR. Genomic DNA of *N. langsdorffii* was digested with *Xba*I and self-ligated with T4 DNA ligase (Takara) to form circular DNA fragments. The circularized DNA was amplified by using two primer sets specific to *NlanCYP82E1*. The PCR products were cloned, and sequenced to verify their identity as *NlanCYP82E1*. All the PCR primers used in cloning *CYP82E* genomic fragments are listed in Table S2.

Genotyping by capillary electrophoresis

PCR primers were designed to obtain distinct intron length polymorphisms for each *CYP82E* gene (Table S3). The 5′-end of the forward primers was fluorescently labeled with 6-FAM (Invitrogen) and VIC (Applied Biosystems). PCR conditions were 5 min at 94°C; 8 cycles of touchdown PCR (30 s at 94 °C; 30 s at 64 °C, with 1°C drop after each cycle; 1min at 72 °C), followed by 32 cycles of regular PCR (30 s at 94°C; 30 s at 57 °C; 1 min at 72 °C), with a final extension of 7 min at 72 °C. For *NalaCYP82E4*, the annealing temperature of touchdown PCR was set at 66°C. The M13-tail PCR method (Schuelke 2000) was used to amplify the intron of *NalaCYP82E3*. The unlabelled forward primer contained a universal M13 sequence at the 5' end, while the universal M13 primer was labeled with VIC. The annealing temperature of touchdown PCR was set at 60°C.

Fluorescently labeled PCR products (1 μL) were mixed with 8.5 μL of Hi-Di formamide (Applied Biosystems) and 0.5 μL of the size standard markers (Applied Biosystems), which contained 68 single-stranded labeled fragments in the range from 20 to 1,200 bp. Samples were denatured by heating for 5 min at 95°C, cooled on ice for at least 3 min, and then applied to an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) equipped with 50-cm capillaries, containing POP-6 polymer. The results were analyzed by GeneMapper Software version 4.0 (Applied Biosystems).

Derived cleaved amplified polymorphic sequences (dCAPS)

The dCAPS procedure was based on the published protocol (Neff et al. 1998). PCR primers were designed to detect single nucleotide polymorphisms in the *CYP82E* genes using the dCAPS Finder 2.0 program (http://helix.wustl.edu/dcaps/dcaps.html), and are listed in Table S4. PCR-amplified products (5 μl) were digested with designated restriction enzymes in a total volume of 15 μl at 37°C overnight, and then analyzed by electrophoresis on a 4% (w/v) NuSieve GTG Agarose gel (FMC BioProducts).

Heterologous expression of CYP82E in yeast

The *Saccharomyces cerevisiae* strain WAT11 (*MAT a*; *ade* 2-1; *his* 3-11, -15; *leu* 2-3, -112; can^R; cyr^+) which expresses *Arabidopsis* ATR1 NADPH-P450 reductase and yeast replicative plasmid pYeDP60 (Pompon et al. 1996) were kindly provided by Dr. P. Urban (Centre de Génétique Moléculaire, CNRS, Gif-sur-Yvette, France). Yeast cells were transformed with the vector constructs by using the yeast electroporation method as described (Thompson et al. 1998), cultured, and then induced for expression of CYP82E as described (Pompon et al. 1996).

The protein-coding sequences of *NalaCYP82E*s were amplified using the appropriate

primer sets from corresponding cDNA clones. The protein-coding sequence of *NlanCYP82E* was obtained by joining the coding sequence from the two exons of a genomic fragment by PCR. Each forward PCR primer contained the *BamH*I restriction site, and the yeast Kozak sequence (Hamilton et al., 1987), whereas each reverse primer contained the *EcoR*I restriction site after the stop codon. The amplified fragments were double digested with *BamH*I and *EcoR*I, and then inserted into the pYeDP60. Primer sequences are listed in Table S2.

After induction of CYP82E, yeast cells were harvested by centrifugation and microsomes were isolated by using enzymatic breaking procedure basically as described (Pompon et al. 1996) with some modifications. Yeast cells were treated with zymolyase (Zymolyase 20T; Seigakaku Corp., Tokyo) at 10 mg g^{-1} at 30˚C with shaking at 90 rpm for 1.5 h. The spheroplasts were lysed by sonication, and the lysate was centrifuged at 20,000 g for 20 min at 4˚C. Subsequently, supernatant was centrifuged at 100,000 g for 1 h at 4˚C, and the microsome pellet was re-suspended in the buffer (20 % glycerol, 1 mM EDTA, 1 mM DTT, and 50 mM Tris-HCl, pH 7.5). Protein concentration was determined by the Coomassie Protein Assay Kit (Thermo Scientific, U.S.).

In vitro enzyme assays for nicotine *N*-demethylase

The nicotine *N*-demethylase activity was measured in a reaction mixture of 100 μL containing 50 mM Tris-HCl buffer (pH 7.5), 1 mM NADPH, 50 μg of microsomes, and (*S*)-nicotine in various concentrations. The assays were initiated with the addition of NADPH. The enzyme activity was determined for 15 min at 25˚C by measuring the consumption of NADPH at 340 nm by the microplate reader (SpectraMax Plus384 Absorbance Microplate Reader, Molecular Devices, U.S.) in triplicate on flat-bottom 96 Ill plates. The sample absorbance values were then subtracted against the values taken from control reactions

without substrate. Kinetic constants were calculated by linear regression of the double-reciprocal analysis, by using GraphPad Prism 5 software (GraphPad Software, U.S.).

Results

Nornicotine formation

Since *N. alata* accumulates tobacco alkaloids exclusively in the root organ and does not transport the alkaloids synthesized in the root to the aerial parts (Pakdeechanuan et al. 2012), I focused on the tobacco alkaloids present in the root. In the root of 10-week-old *N. alata* plants, nicotine was the major alkaloid at the concentration of 4.07 mg g dry weight^{-1}, followed by nornicotine (2.83 mg g dry weight^{-1}) and anatabine (1.81 mg g dry weight^{-1}) (Table 1). The conversion rate (%) from nicotine to nornicotine was calculated by dividing the nornicotine content by the sum of the nicotine content and the nornicotine content, which in the case of the *N. alata* root was 41.0%, showing converter phenotype. However, I only found nicotine (4.33 mg g dry weight^{-1}) and anatabine (0.61 mg g dry weight^{-1}) in the root of 10-week-old *N. langsdorffii* plants; no nornicotine was detectable, suggesting non-converter phenotype. The root of the F_1 hybrid plants between *N. alata* and *N. langsdorffii* showed a nornicotine conversion rate of 16.6%, an intermediate between the two parental values (Table 1 and Figure 12A), indicating that nornicotine formation is a semi-dominant trait.

Table 1. Alkaloid contents in the roots of *N. alata*, *N. langsdorffii*, and their F_1 hybrid.

Plants	Alkaloid content (mg g dry weight^{-1})			Conversion
	Nicotine	Nornicotine	Anatabine	(%)
N. alata	4.07 ± 3.26	2.83 ± 2.22	1.81 ± 0.64	41.0
N. langsdorffii	4.33 ± 0.74	0	0.61 ± 0.61	0
F_1	2.27 ± 0.51	0.45 ± 0.21	0.28 ± 0.19	16.6

To examine genetic loci involved in nornicotine formation, I first analyzed nornicotine conversion rates in the root of 109 individual F_2 plants derived from the self-pollination of the F_1 hybrid plants (Figure 12B). Among these plants, 26 plants (22.9 %) did not contain nornicotine and were classified as non-converters, whereas the rest of the plants were classified as converters. Thus, the segregation ratio observed for nicotine to nornicotine conversion trait showed 83 converter plants versus 26 nonconverter plants that fitted the expected segregation ratio of 3:1 (Table S5), indicative of a single (semi)dominant locus. Moreover, the converter plants showed different degrees of nicotine conversion that ranged from very low conversion levels to levels greater than those observed in *N. alata*. In that case, the variation in the percentage of nicotine conversion occurring in the segregating populations could be explained by complicated genetic mechanisms involving quantitative effects. The observed results indicated that a single semi-dominant locus controls nornicotine formation.

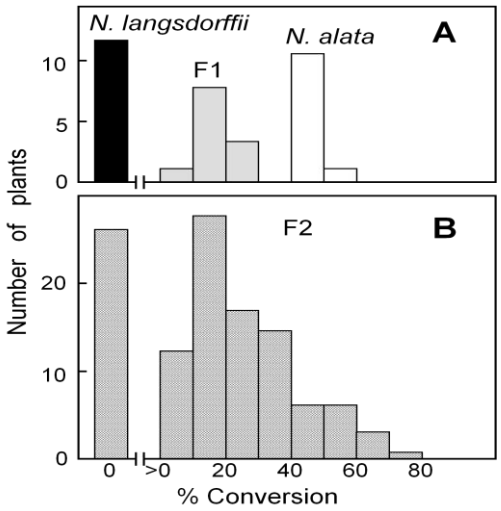

Figure 12. Frequency distributions of the nicotine-to-nornicotine convertion rate (%) in the roots of the 10-week-old plants. (A) *N. alata*, *N. langsdorffii*, and their F_1 hybrid. (B) F_2 progeny.

CYP82E* genes in *N. alata* and *N. langsdorffii

Because nicotine *N*-demethylase is encoded by cytochrome P450 monooxygenases of the CYP82E subfamily in *N. tabacum* (Siminszky et al. 2005; Lewis et al. 2010), I extensively amplified genomic DNA fragments from *N. alata* and *N. langsdorffii* by using the PCR primers designed from the tobacco *CYP82E* sequences, and finally obtained four *CYP82E* genes from *N. alata* (*NalaCYP82E1*, *NalaCYP82E2*, *NalaCYP82E3*, and *NalaCYP82E4*) and two from *N. langsdorffii* (*NlanCYP82E1* and *NlanCYP82E2*). To distinguish between nicotine *N*-demethylase genes from *N. alata* and *N. langsdorffii*, an *Nala* or *Nlan* prefix is attached, respectively, to the name of the genes in my observation.

Semi-quantitative RT-PCR results were consistent with the previous RNA gel blot analysis in Chapter I that expression of *CYP82E genes of N. alata* was preferentially expressed in roots and green leaves whereas the expression level of *CYP82E* genes from *N. langdorffii* presented at very low level (Figure 13). Reverse transcription-PCR was next used to clone full-length cDNA clones for these *CYP82E* genes, except that no cDNA clones corresponding to *NlanCYP82E1* were obtained even after cDNA pools were prepared from several different tissues of *N. langsdorffii*. Their DNA sequences have been deposited in the GenBank: *NalaCYP82E1* (AB709932), *NalaCYP82E2* (AB709933), *NalaCYP82E3* (AB709934), *NalaCYP82E4* (AB709935), *NlanCYP82E1* (AB709936), and *NlanCYP82E2* (AB709937).

Based on alignment of the genomic sequences and the cDNA sequences, the exon-intron structure of the *CYP82E* genes was determined (Figure 14). The open reading frame of *NlanCYP82E1*, for which no cDNA was available, was deduced by comparison with other *CYP82E* sequences. The *CYP82E* genes of *N. alata* and *N. langsdorffii* were composed of two highly conserved exons, separated by a less conserved intron with varying length, and encoded proteins highly similar in amino acid sequences to CYP82Es of *N. tabacum*, *N.*

sylvestris, and *N. tomentosiformis* (Figure 15; Siminszky et al. 2005, Gavilano et al. 2007, Chakrabarti et al. 2008, Gavilano and Siminszky 2007, Lewis et al. 2010). The number and the position of intron are conserved in *CYP82E4* of *N. tabacum* (Xu et al. 2007) and *CYP82E2* of *N. sylvestris* (Chakrabarti et al. 2007).

Interestingly, I found that a nucleotide at the 345th from the first ATG is deleted in the first exon of *NlanCYP82E2*, which caused frame shift in the downstream coding sequence and resulted in a truncated CYP82E protein (Figure 14, and Figure S1). If cytosine is inserted into the deleted nucleotide position, the restored NlanCYP82E2 protein shows very high similarity to other CYP82Es of *N. alata* and *N. langsdorffii*.

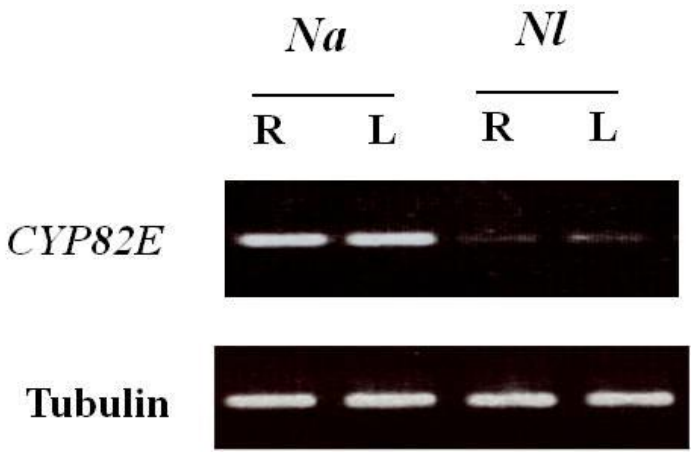

Figure 13. Semiquantitative RT-PCR analysis of *CYP82E* gene transcripts in roots and leaves of *N. alata* and *N. langsdorffii*. Tubulin transcripts were analyzed as a control. R, root; L, leaf; *Na*, *N. alata*; *Nl*, *N. langsdorffii*.

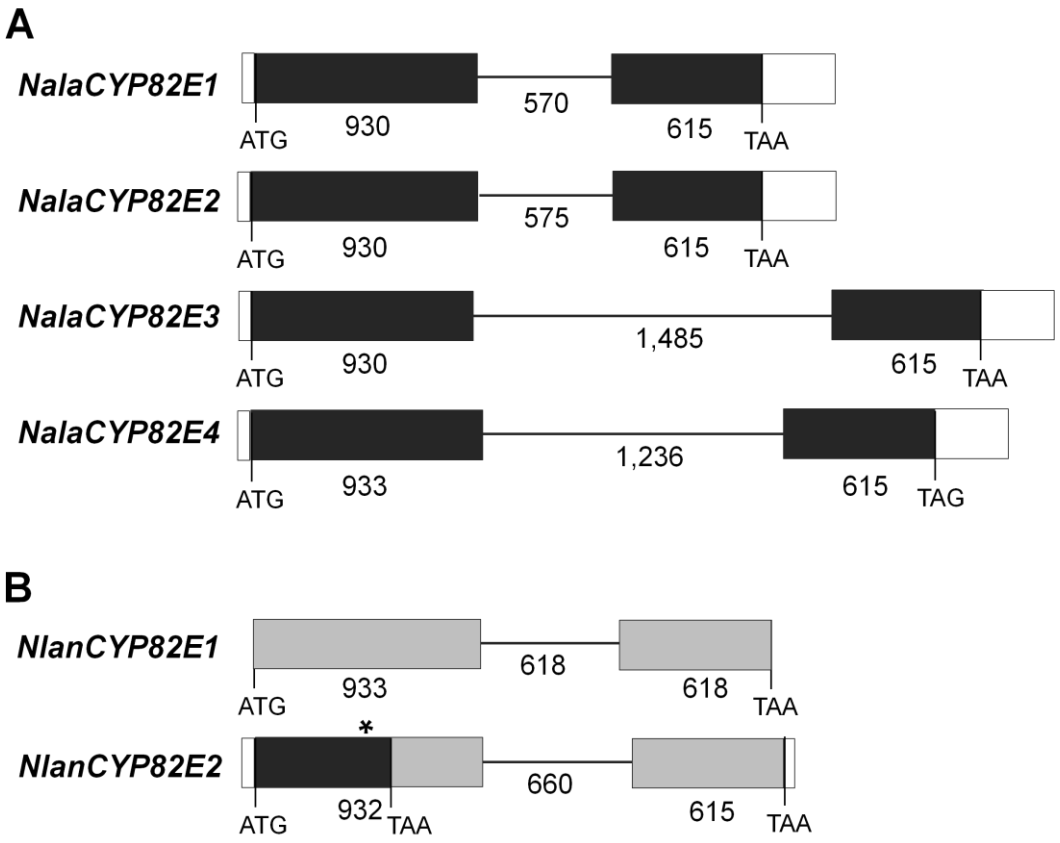

Figure 14. Organization of *CYP82E* genes. The 5'- and 3'-untranslated regions are shown in open boxes, whereas the filled boxes indicate the protein coding regions, and have the numbers indicating their length in bp. Introns are shown in lines, with their length (bp) indicated below. (A) Four *CYP82E* genes in *N. alata*. (B) Two *CYP82E* genes in *N. langsdorffii*. The gene organization of *NlanCYP82E1* was deduced from the structures of other *CYP82E* genes. A single nucleotide deletion (asterisk) in the first exon of *NlanCYP82E2* causes a frame shift and creates a premature stop codon. The grey regions of the exons indicate the predicted protein coding regions if a full-length CYP82E protein is restored by an insertion of the deleted nucleotide.

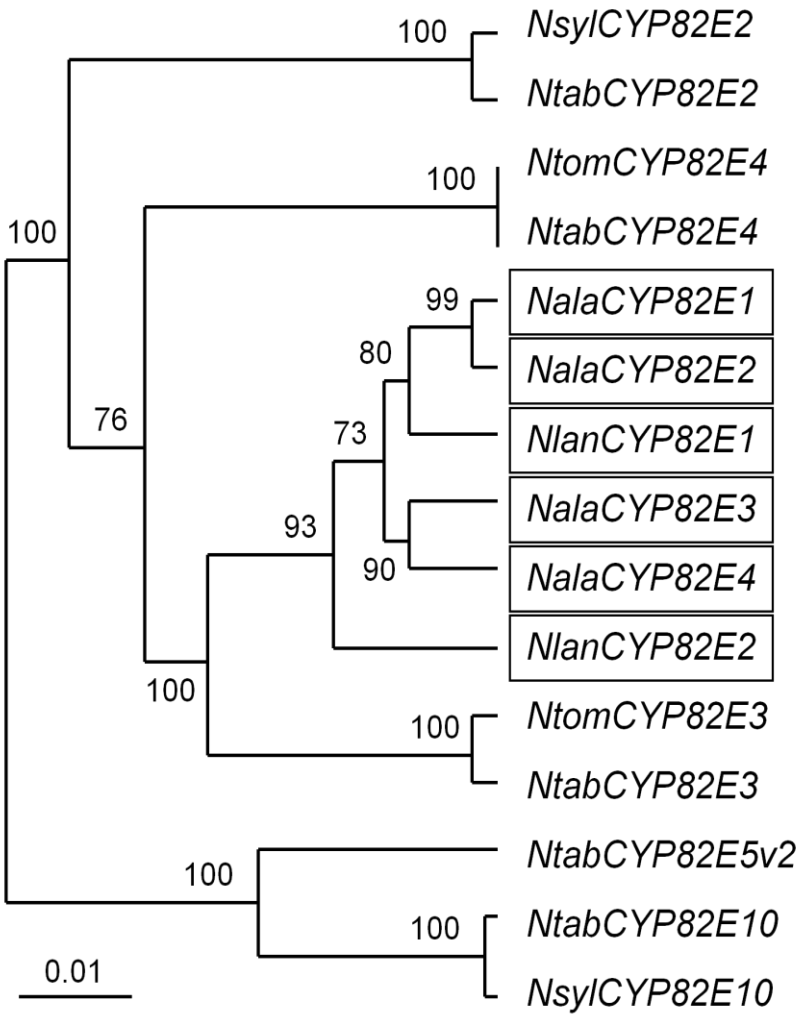

Figure 15. Phylogenetic relationship of CYP82Es. The CYP82E sequences reported in this study are boxed. The restored NlanCYP82E2 sequence is used. The tree was constructed by the UPGMA method based on a distance matrix calculated by the ClustalW program. The number at each node indicates the bootstrap value based on 100 samplings. The scale bar indicates the distance corresponding to 1 difference per 100 positions. *Nala*; *N. alata*, *Nlan*; *N. langsdorffii*, *Nsyl*; *N. sylvestris*, *Ntab*; *N. tabacum*, and *Ntom*; *N. tomentosiformis*.

***N. alata* CYP82Es and *N. langsdorffii* CYP82E1 are functional nicotine *N*-demethylases**

(this subsection was done by Mr. Seddon Teoh)

Since a few degenerative mutations in the CYP82E sequences result in inactivation of nicotine *N*-demethylase (Chakrabarti et al. 2007, Lewis et al. 2010), Mr. Teoh examined whether the *CYP82E* genes found in this study encode functional enzymes. For *NlanCYP82E1*, for which no cDNA was available, a full length cDNA by removing the intron from the genomic DNA fragment was constructed. Four full length *NalaCYP82E* cDNAs and the constructed *NlanCYP82E1* cDNA were individually expressed in yeast strain WAT11, which co-expressed an *Arabidopsis* P450 reductase gene (Pompon et al. 1996). Microsomal fractions isolated from yeast cells that expressed any one of the five *CYP82E* clones catalyzed the conversion of nicotine to nornicotine (Figure 16A). Detailed catalytic data showed that these nicotine *N*-demethylases possess high affinity toward nicotine, with *K*m values for nicotine ranging from 7.7 μM to 32.7 μM (Figure 16B). These *K*m values are comparable to those reported for other functional *Nicotiana* CYP82E enzymes expressed in yeast (Gavilano et al. 2007, Gavilano and Siminszky 2007, Xu et al. 2007, Lewis et al. 2010). The results indicated that *NalaCYP82E* genes and *NlanCYP82E1* encode functional enzymes.

***NlanCYP82E1* is not expressed due to defects in *cis*-activation**

Because *NlanCYP82E1* encodes functional nicotine *N*-demethylase, nornicotine formation in *N. langsdorffii* critically depends on whether this gene is expressed or not. The previous RNA gel blot analysis of the *CYP82E* transcripts by using an *N. tabacum CYP82E4* probe (Figure 4) and semi-quantitative RT-PCR (Figure 13) indicated that *CYP82E* genes are expressed strongly in the root of *N. alata* but scarcely in the root of *N. langsdorffii*. In this study, I amplified *CYP82E* cDNAs from the root of *N. langsdorffii* by using a common PCR primer set which would anneal perfectly to all six *CYP82E* genes identified in this study, and

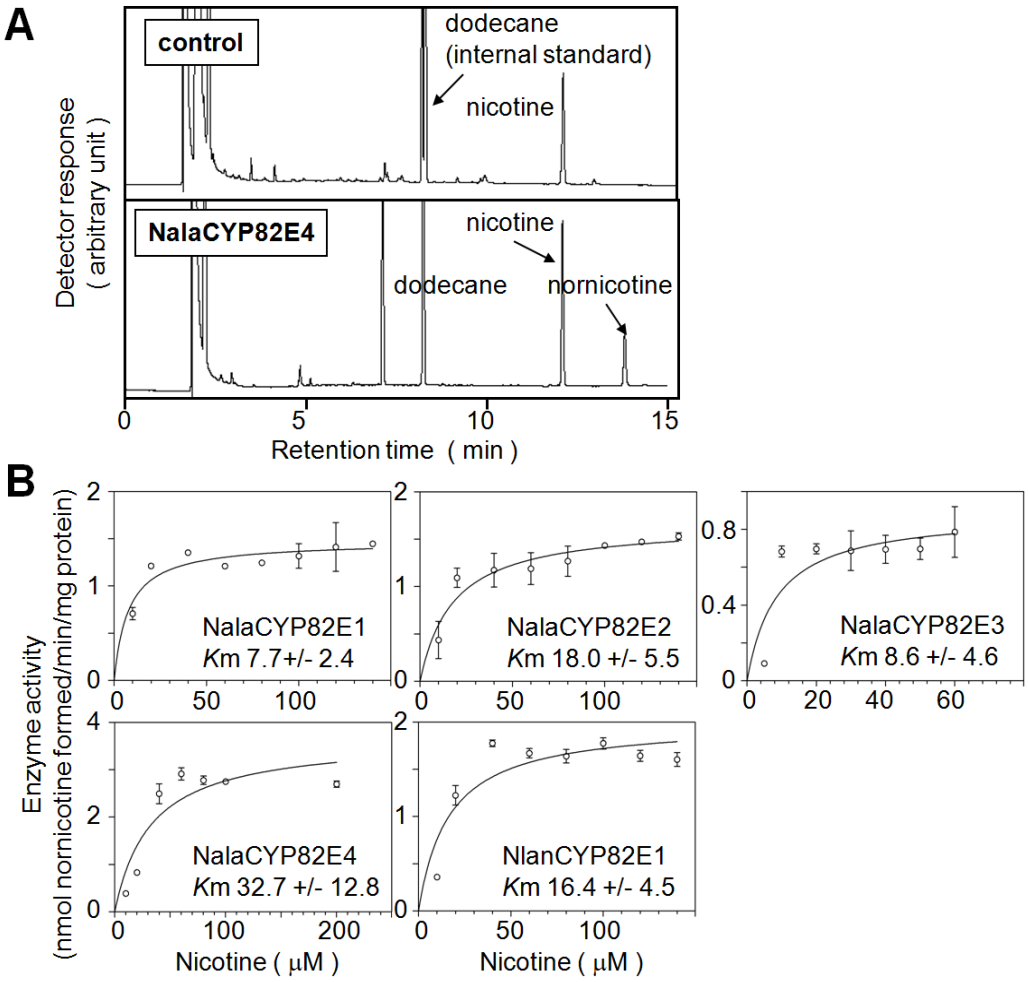

Figure 16. CYP82Es from *N. alata* and *N. langsdorffii* are functional nicotine *N*-demethylases. Microsomes were prepared from the yeasts expressing recombinant CYP82E proteins, and were used for the nicotine *N*-demethylase assays. (A) Gas-liquid chromatograms of the reactions products. Microsomes were prepared from the control yeasts expressing an empty vector (control). Dodecane was included in the samples as an internal standard. (B) Nornicotine formation as a function of nicotine as substrate. The *K*m values for nicotine (in μM) were calculated from these plots, and are indicated as the means +/- the standard deviations.

* This figure is based on the results obtained by Mr. Seddon Teoh.

then sequenced 100 individual cDNA clones to reveal their identity. All sequenced clones were found to be *NlanCYP82E2*; no *NlanCYP82E1* clone was present (Figure 17B), which is consistent with my inability to obtain any *NlanCYP82E1* cDNAs by reverse transcription-PCR based on its genomic sequence information. Similar expression analysis of *N. alata* indicated that all four *NalaCYP82E* genes are expressed in the root of *N. alata* (Figure 17A).

The loss of expression of *NlanCYP82E1* might result from *cis*- and/or *trans*-regulatory changes. F_1 hybrids provide a useful tool to distinguish between differences in *cis*- or *trans*-activation of species-specific alleles of a gene (Wittkopp et al. 2004). Differences in *trans*-regulatory activity can be inferred by comparing the ratio of allelic expression in hybrids with the ratio of gene expression between parent species, which has been used, for example, to analyze the expression difference of a transcription factor gene for floral scent production in two *Petunia* species (Klahre et al. 2011).

As described in Figure S2, *cis*- and *trans*-regulatory mutations can be distinguished by the presence or absence of *NlanCYP82E1* transcripts in the F_1 genetic background, where both parental alleles share the same *trans*-regulatory factors. The hypothesis is that if no *NlanCYP82E1* mRNA is detected in the F_1 genetic background, a *cis*-regulatory change results in the absence of expression of *NlanCYP82E1* in *N. langsdorffii*, whereas, in the case of a *trans*-regulatory change, *NlanCYP82E5* mRNA is observed in the F_1 genetic background due to the influence of shared *trans*-regulatory elements.

Breakdown of the *CYP82E* cDNAs prepared from the root of four F_1 hybrid plants between *N. alata* and *N. langsdorffii* revealed that *NalaCYP82E* clones predominated, whereas *NlanCYP82E2* clones represented only 3% and *NlanCYP82E1* was absent (Figure 17C), indicating a defect in the *cis*-regulatory activity. The nonsense-mediated mRNA decay pathway, which targets mRNA with premature termination codons for rapid

degradation (Rebbapragada and Lykke-Andersen 2009), may also contribute to a low abundance of *NlanCYP82E2* mRNA. In my experiments, the ratios of *NalaCYP82E1*, *NalaCYP82E2*, and *NalaCYP82E3* clones were similar between *N. alata* and the F_1 hybrids, while the ratio of *NalaCYP82E4* was considerably different between the two samples. Likely cause of this difference will be discussed below.

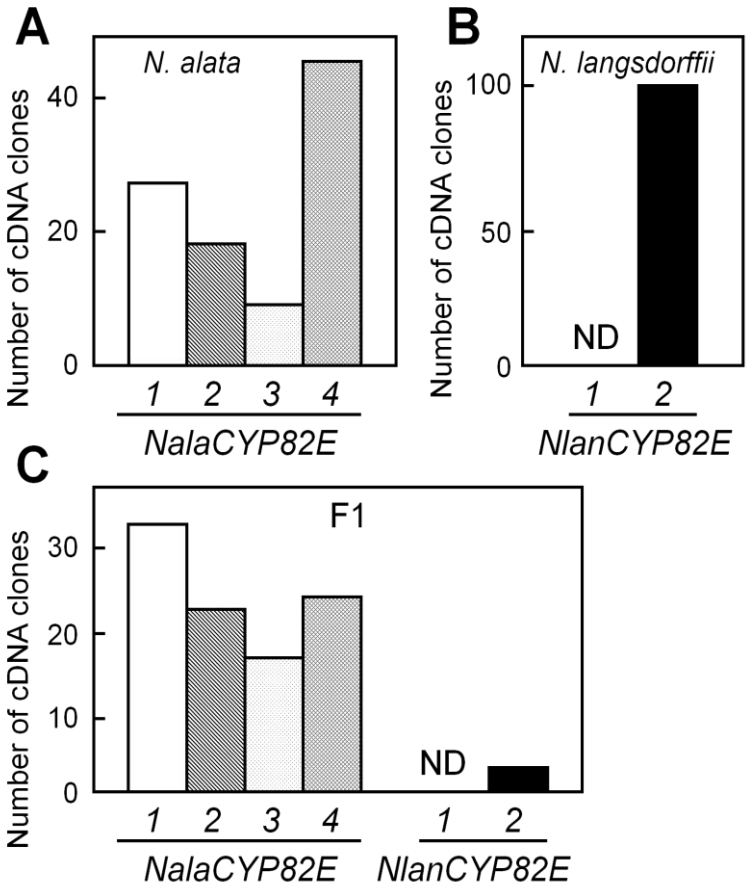

Figure 17. Expression levels of *CYP82E* genes in the roots of 10-week-old plants. *CYP82E* cDNA fragments were amplified from the RNA samples, which had been prepared from the roots of four independent plants. One hundred *CYP82E* cDNA clones were sequenced each from *N. alata* (A), *N. langsdorffii* (B), and their F_1 hybrid (C), and grouped into the *CYP82E* family members. ND; not detected.

Segregation of *CYP82E* genes in the F_2 progeny

To examine presence or absence of the six *CYP82E* genes in the individual F_2 plants, which were derived from self-pollination of more than 10 F_1 plants, I developed a capillary electrophoresis-based analytical system that detected fragment length polymorphism of these *CYP82E* introns (Figure 18A; see Materials and Methods). Genomic DNA samples from *N. langsdorffii* gave PCR fragments of 690 bp and 726 bp, which respectively corresponded to *NlanCYP82E1* and *NlanCYP82E2*, whereas *N. alata* DNA samples gave PCR fragments of 369 bp (*NalaCYP82E3*), 603 bp (*NalaCYP82E4*), 637 bp (*NalaCYP82E1*), and 642 bp (*NalaCYP82E2*). In the chromatogram of an F_1 hybrid sample, all the six *CYP82E* fragments were clearly detectable and were well separated.

With this method, I next analyzed segregation of *CYP82E* genes in 109 F_2 plants (Table S1). When fragment peaks were ambiguous, I confirmed the genotype of these samples by standard genomic PCR amplification of the *CYP82E* genes and the derived cleaved amplified polymorphic sequences (dCAPS) assay (Figure S3; see Materials and Methods). *NalaCYP82E1*, *NalaCYP82E2*, *NalaCYP82E3*, *NlanCYP82E1*, and *NlanCYP82E2* were present in approximately 75% of the F_2 plants, as expected for a simple Mendelian segregation ($p<0.05$), whereas *NalaCYP82E4* was inherited only in 27.5% of the F_2 population (Table 2). To explain this unexpectedly low segregation frequency, I explored a possibility that *NalaCYP82E4* is present only in some, but not all, of the *N. alata* plant population used in my experiment, and that some F_1 plants did not inherit this gene. Indeed, when twelve *N. alata* individuals were tested for the presence of *NalaCYP82E4* by genomic PCR analysis, I found this gene in eight plants; the remaining four plants did not have it (Figure S3). Because DNA samples of the F_1 plants were not available at this stage, I could not directly test whether some F_1 plants did not inherit *NalaCYP82E4*. In conclusion, heterogeneous occurrence of *NalaCYP82E* in the *N. alata* plants used in this study appears to

have caused an abnormal frequency of this gene in the F_2 segregation analysis.

Table 2 Allele frequencies of *CYP82E* genes among the F_2 plants

Phenotype[a]	*n*	Number of plants[b]					
		NalaE1	*NalaE2*	*NalaE3*	*NalaE4*	*NlanE1*	*NlanE2*
Non-converter	26	0	0	0	0	26	26
Medium converter	73	71	70	69	24	60	54
High converter	10	9	10	9	6	0	0
Total	109 (100%)	80 (73.4%)	80 (73.4%)	78 (71.6%)	30 (27.5%)	86 (78.9%)	80 (73.4%)

[a]Non-converters did not contain detectable amounts of nornicotine, whereas low converters and high converters showed nicotine-to-nornicotine conversion rates of less than 50% and conversion rates exceeding 50%, respectively.

[b]The number of plants which contained the indicated *CYP82E* alleles. The*CYP82E* genes were shown in abbreviated forms. *NalaE1*, for example, indicates *NalaCYP82E1*.

I next analyzed co-segregation of the *CYP82E* genes. The linkage analysis indicates that *N. alata CYP82E* genes are linked, and are aligned in a chromosomal region with a genetic distance of 5.5 cM between *NalaCYP82E1* and *NalaCYP82E2*, and with 1.8 cM between *NalaCYP82E2* and *NalaCYP82E3* (Figure 18B). In a *N. langsdorffii* chromosomal region, *NlanCYP82E1* and *NlanCYP82E2* are linked with a distance of 5.5 cM.

Finally, I examined the relationship between phenotype (nornicotine accumulation) and genotype (*CYP82E*) (Table S1). F_2 plants were classified into non-converters (no nornicotine detected), medium converters (less than 50% conversion), and high converters (conversion rates exceeding 50%) (Figure 12), and allele frequencies of *CYP82E* were scored

for each group (Table 2). Non-converters (n = 26) did not possess any of the four *N. alata CYP82E* genes, but instead had both *CYP82E* genes of *N. langsdorffii*. In medium converters (n = 73), two or more *N. alata CYP82E* genes were always present, and *NlanCYP82E1* and *NlanCYP82E2* were found in most plants. High converts (n = 10) also contained two or more *N. alata CYP82E* genes but *NlanCYP82E1* and *NlanCYP82E2* were absent altogether. These genetic data indicates that the clustered *CYP82E* genes of *N. alata* and *N. langsdorffii* are located on the corresponding locus of the chromosome in these two diploid *Nicotiana* species. Whereas the *N. alata CYP82E* genes act semi-dominantly to individually enhance conversion of nicotine to nornicotine, the *N. langsdorffii CYP82E* genes are non-functional.

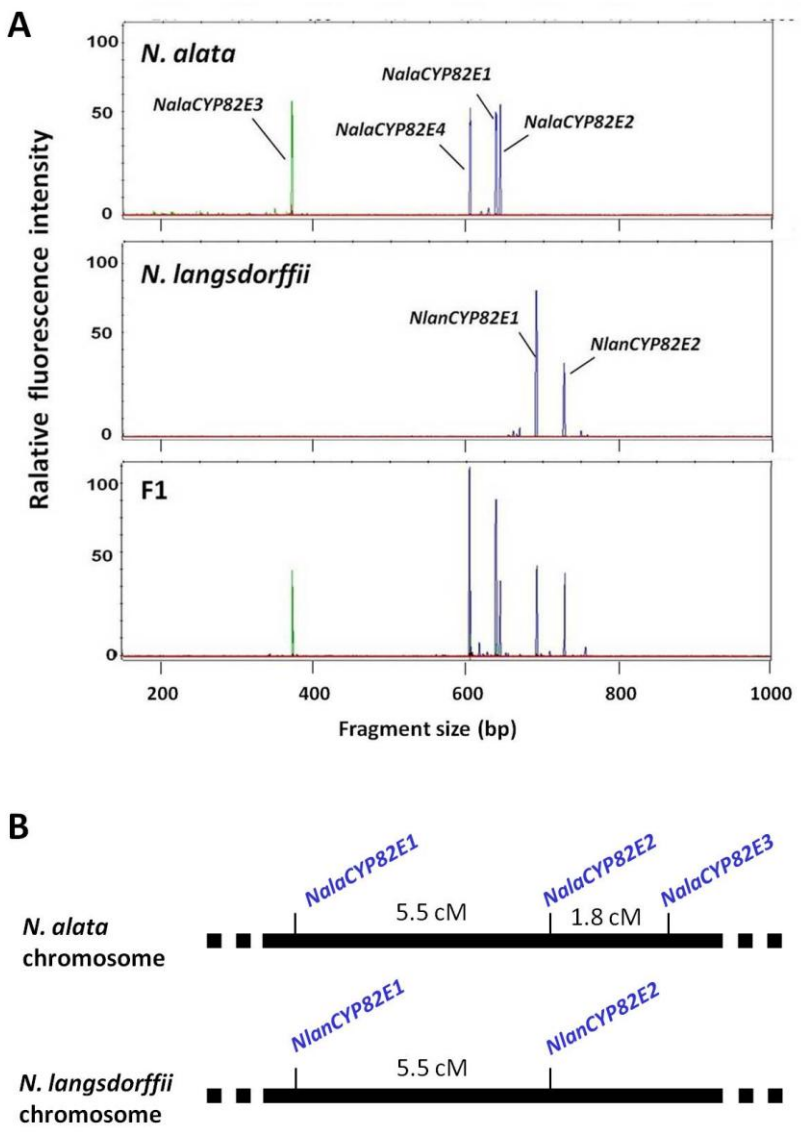

Figure 18. Segregation analysis of *CYP82E* genes in 109 F_2 progeny plants. (A) Capillary electrophoresis-based fragment length polymorphism analysis of the *CYP82E* introns in *N. alata*, *N. langsdorffii*, and their F_1 hybrid. DNA size is shown in base pair in the *x*-axis, while the *y*-axis indicates relative fluorescence intensity. Amplified DNA fragments showed the expected sizes; *NalaCYP82E1* (637 bp), *NalaCYP82E2* (642 bp), *NalaCYP82E3* (369 bp), *NalaCYP82E4* (603 bp), *NlanCYP82E1* (690 bp), and *NlanCYP82E2* (726 bp). (B) Genetic linkage maps of *CYP82E* genes in *N. alata* and *N. langsdorffii*. Segregation data for *NalaCYP82E4* was not included in the analysis.

Discussion

In this study, I provided genetic, molecular, and biochemical evidence that *CYP82E* genes encoding functional nicotine *N*-demethylases are responsible for accumulation of nornicotine in *N. alata*. In *N. langsdorffii* where two *CYP82E* genes were inactivated and in the F_2 progeny plants which did not inherit functional *CYP82E* genes from *N. alata*, nornicotine was below the detection limit of my sensitive capillary gas liquid chromatography assay. An *N*-methylputrescine oxidase-mediated and nicotine-independent pathway of nornicotine formation (Heim et al. 2007; Katoh et al. 2007) has been postulated to explain residual amounts of nornicotine in the triple tobacco mutants for *CYP82E* (Lewis et al. 2010), but such a bypass pathway, if exists in planta, does not contribute to nornicotine formation in *N. alata*. Moreover, conversion of nicotine to nornicotine in plants appeared to be dependent on the dosage of functional *CYP82E* genes; F_1 hybrid plants showed approximately half the conversion rate of *N. alata*, and the low converters in the F_2 progeny possessed both *N. alata* and *N. langsdorffii CYP82E* alleles, indicative of the heterozygosity of the functional *N. alata* alleles. Thus, nicotine *N*-demetylase activity is rate-limiting for nornicotine formation in *N. alata* and its hybrid progenies.

Modern allotetraploid tobacco (*N. tabacum*) generally does not accumulate nornicotine, whereas its progenitors (*N. sylvestris* and *N. tomentosiformis*) convert nicotine to nornicotine in the senescing leaf (Wernsman and Matzinger 1968). In tobacco, three *CYP82E* genes are inactivated. The nicotine *N*-demethylase activity of the *CYP82E2* gene derived from *N. sylvestris* is inactivated by the E375K and W420L mutations (Chakrabarti et al. 2007), whereas the *N. tomestosiformis*-originated *CYP82E3* gene encodes a nonfunctional enzyme due to the W330C mutation (Gavilano et al. 2007). The *CYP82E4* gene from *N. tomentosiformis* is transcriptionally silenced. Transcriptional silencing of *CYP82E4* in

tobacco is unstable, and this gene is often reactivated to produce converter tobacco lines. Allopolyploidization is associated with transcriptional silencing of a considerable proportion of the transcriptome, and may be caused by epigenetic changes, transposon activation, and small RNA- and RNA-interference-mediated interactions (Adams and Wendel 2005). Transcriptional inactivation mechanism of *CYP82E4* in tobacco may be distinct from that observed in this study since inactivation of *NlanCYP82E1* is stable and is not triggered by polyploidization. Loss of critical transcription factor-binding sites in the *NlanCYP82E1* promoter might underlie the *cis*-inactivation of this gene.

In *N. tomentosiformis,* the conversion locus contains at least two nicotine *N*-demethylase genes, *NtomCYP82E3* and *NtomCYP82E4* (Gavilano et al. 2007, Chakrabrti et al. 2007). In this study, I showed that *NalaCYP82E1*, *NalaCYP82E2*, and *NalaCYP82E3* are clustered within a 7.3-cM chromosomal interval of *N. alata*, while *NlanCYP82E1* and *NlanCYP82E2* are linked at a genetic distance of 5.5 cM. Therefore, duplication of *CYP82E* genes at the conversion locus appears to be a general feature in diploid *Nicotiana* species. Duplicated genes may undergo rapid diversification in the expression profile or protein function (Lynch and Conery 2000). *NtomCYP82E3* is expressed strongly in the green tobacco leaf and is downregulated during senescence, whereas expression of *NtomCYP82E4* is low in the green leaf and is strongly induced during senescence (Gavilano et al. 2007). The four *N. alata CYP82E* genes may be expressed in distinct patterns during growth, development, and in responses to environmental stimuli, but I did not analyze expression patterns of each *CYP82E* gene because of the difficulties in designing appropriate PCR primers in the quantitative reverse transcription PCR assay.

N. alata is strongly self-incompartible, and usually propagates by out-crossing. In such an out-crossing species, a plant population may exhibit high polymorphic variation at many genetic loci. I found that *NalaCYP82E4* is present in only some of the *N. alata* plants

in my population, and suspect that the *NalaCYP82E4* DNA sequence represents a polymorphic allele of *NalaCYP82E4*. My PCR primers used to amplify *NalaCYP82E4* probably fail to detect other *NalaCYP82E4* alleles that may be present in the plant population used in this study. Results involving *NalaCYP82E4* (Figure 17, and Table 2) are complicated by this polymorphism.

I conclude that *CYP82E* genes encoding nicotine *N*-demethylase are duplicated at closely linked chromosomal regions in the two diploid *Nicotiana* species of the Alatae section, and that the two *CYP82E* genes in *N. langsdorffii* are made nonfunctional by either transcriptional inactivation or immature translational termination, resulting in elimination of nornicotine in this species. While many tobacco herbivores show higher toxicity toward nicotine than nornicotine, some insects are more susceptible to nornicotine (Siegler and Bowen 1946, Soloway 1976). Contrasting ratios of these tobacco alkaloids may influence interactions with visiting herbivores to *N. alata* and *N. langsdorffii*.

Future Perspectives

In my doctoral dissertation work, I chose two closely-related wild *Nicotiana* species to study how composition and long-range transport of tobacco alkaloids are controlled. The two *Nicotiana* species were selected because they can be sexually crossed to produce viable progenies and showed characteristic alkaloid profiles. Such hybrid *Nicotiana* plants have been previously used to study morphological traits, but my studies demonstrate that they can be also useful in studies of more complex traits, such as whole plant distribution of natural products.

Recent molecular studies identified several transporters involved in subcellular localization of natural products, including tobacco alkaloids. However, inter-organ translocation of natural products remains a least understood process, and will certainly be approached by genetic studies. Since my work identified that the failure in root-to-shoot translocation in *N. alata* is controlled by a single major dominant locus, the next step will be genetically fine-map the locus and then to molecularly clone the responsible gene(s). cDNA microarrays and genome sequences, although still fragmentary, are beginning to be available in several *Nicotiana* species, and the time will soon be ripe for identifying the translocation gene(s) and the cellular and biochemical mechanisms involved. I hope that ambitious researcher will succeed my work and will unveil the mystery of the long-range alkaloid transport.

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May, 2012

Phatharapron PAKDEECHANUAN

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SUPPLEMENTARY FIGUREURES

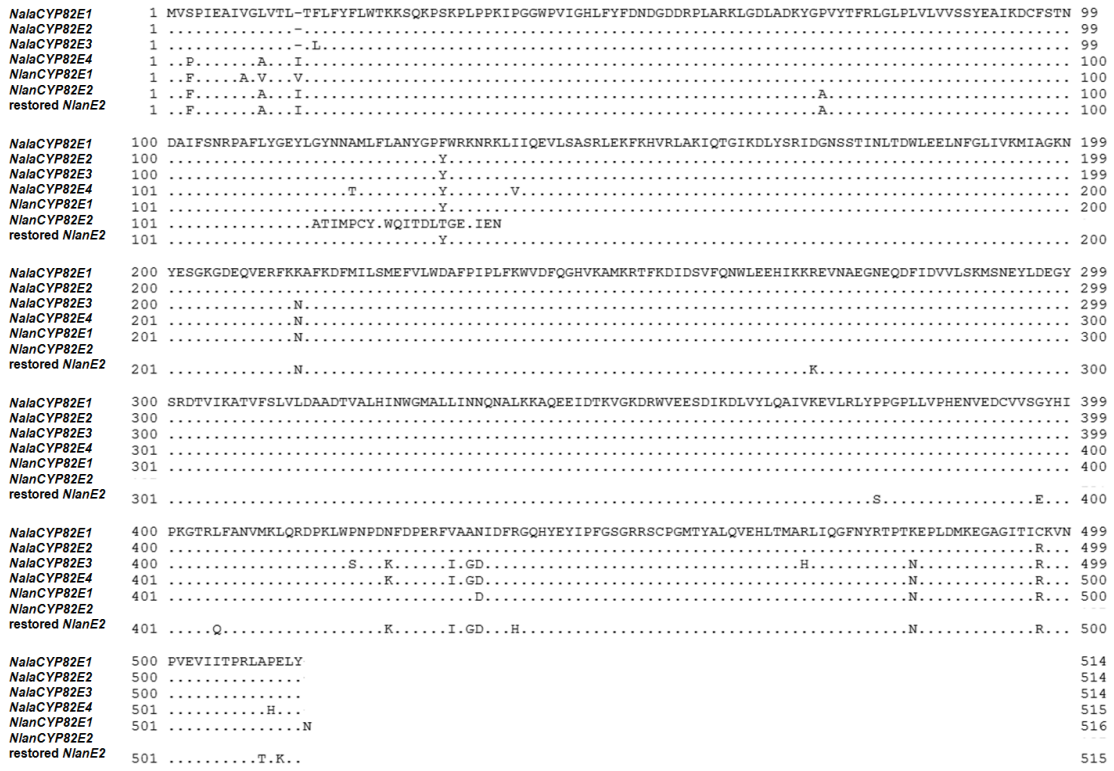

Figure S1. **Alignment of predicted amino acid sequences.** Identical amino acid residues are shown as dots, and the gaps (shown in dashes) are introduced to maximize the alignment. A restored NlanCYP82E2 sequence is generated by inserting the deleted cytosine at the 345th position.

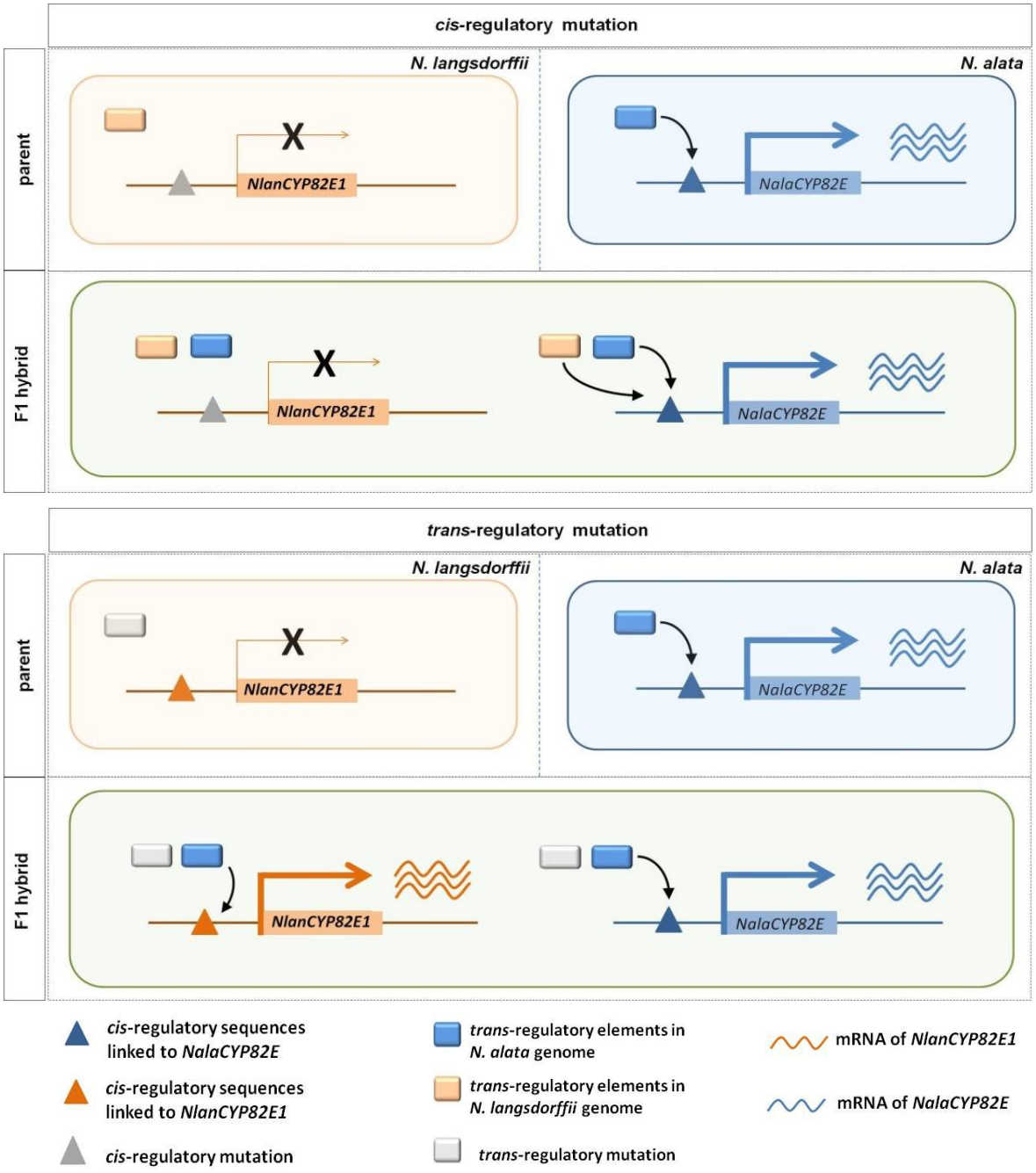

Figure S2. **A model for the detection of *cis*- or *trans*- regulatory changes in *NlanCYP82E1* by observing relative expression of heterozygous alleles from both parents in F_1 hybrids.** *cis*-regulatory factors are usually linked to a gene but *trans*-regulatory factors are located elsewhere in genome. Two hypothetical mechanisms are shown. In the case of *cis*-acting change (upper section), *NlanCYP82E1* is not expressed in the F_1 hybrid genetic background. In the case of *trans*-acting change (lower section), *NlanCYP82E1* is expressed under the influence of shared *trans*-acting factors.

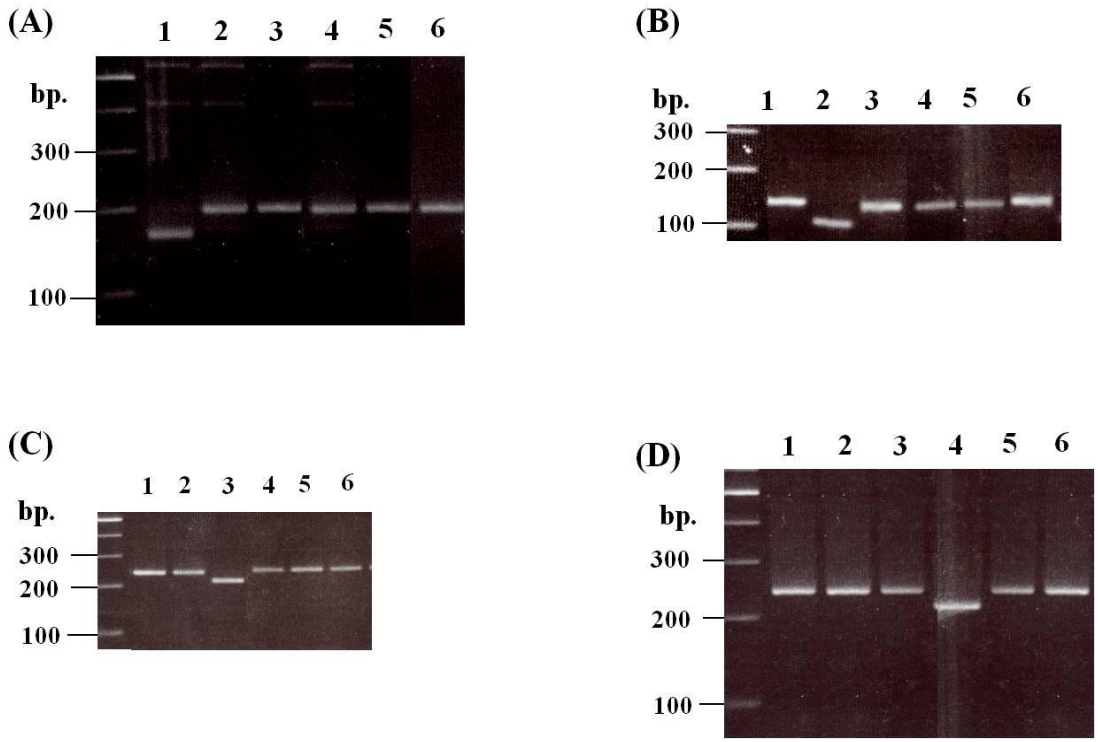

Figure S3. **Detection of each *N. alata CYP82E* gene using dCAPS markers.** DNA size marker (M), PCR products from *NalaCYP82E1* (lane1), *NalaCYP82E2* (lane2), *NalaCYP82E3* (lane3), *NalaCYP82E4* (lane4), *NlanCYP82E1* (lane5) *and NlanCYP82E2* (lane6). (A) Electrophoresis profiles of dCAPS product digested by *Dde*I for screening *NalaCYP82E1*. (B) Electrophoresis profiles of dCAPS product digested by *Sac*I for screening *NalaCYP82E2*. (C) Electrophoresis profiles of dCAPS product digested by *Kpn*I for screening *NalaCYP82E3*. (D) Electrophoresis profiles of dCAPS product digested by *Hind*III for screening *NalaCYP82E4*.

SUPPLEMENTARY TABLES

Table S1 Alkaloid contents and inheritance of CYP82E genes in the F_2 plants

Plant	Alkaloid content in roots (µg/mg DW)			conversion	Fragment Analysis*					
No.	nicotine	nornicotine	anatabine	(%)	*Nala CYP82E1*	*Nala CYP82E2*	*Nala CYP82E3*	*Nala CYP82E4*	*Nlan CYP82E1*	*Nlan CYP82E2*
2	3.3867	0	0.1475	0.00						
4	3.6690	0	0.5483	0.00						
5	5.5472	0	0.2670	0.00						
6	3.8691	0	0.5374	0.00						
9	6.8861	0	1.0791	0.00						
13	2.8805	0	0.5261	0.00						
15	7.8768	0	0.4612	0.00						
19	0.8929	0	0.4640	0.00						
23	4.1371	0	0.3415	0.00						
25	1.8454	0	0.3184	0.00						
29	0.0372	0	0.0076	0.00						
37	1.1118	0	0.1353	0.00						
44	6.0380	0	0.7700	0.00						
48	2.7656	0	0.4974	0.00						
63	3.2852	0	0.3753	0.00						
65	2.3907	0	0.3434	0.00						
84	0.6085	0	0.0540	0.00						
98	0.7997	0	0.0262	0.00						
106	7.5266	0	0.2788	0.00						
107	1.6451	0	0.2557	0.00						
110	2.1626	0	0.2627	0.00						
112	0.1159	0	0.0000	0.00						
114	0.1616	0	0.0000	0.00						
119	5.2840	0	0.0454	0.00						
121	2.3205	0	0.3323	0.00						
125	1.3520	0	0.1689	0.00						
43	3.7170	0.1327	0.4962	3.45						
74	10.9218	0.5805	0.5128	5.05						
33	1.0686	0.0744	0.2706	6.51						
8	5.5137	0.3953	0.5850	6.69						
7	10.7773	0.8119	0.3646	7.01						
30	0.0502	0.0039	0.0366	7.21						
10	6.3624	0.5499	0.6268	7.96						
12	1.5880	0.1379	0.1491	7.99						
24	1.8417	0.1604	0.3436	8.01						
1	3.9494	0.3732	0.3111	8.63						
64	3.5568	0.3619	0.1735	9.23						
96	0.6894	0.0718	0.0419	9.43						
67	7.5476	0.8541	0.2332	10.17						
45	4.7448	0.5437	0.3370	10.28						
21	0.4145	0.0500	0.0694	10.76						
90	4.3418	0.5350	0.1701	10.97						
41	5.5387	0.6907	0.7554	11.09						
32	4.6388	0.5810	0.2583	11.13						
31	1.9151	0.2461	0.2829	11.39						
123	4.3255	0.5647	0.2688	11.55						
95	2.1562	0.2894	0.0765	11.83						
91	2.1811	0.3118	0.1868	12.51						
20	3.5549	0.5262	0.2952	12.89						
47	8.3543	1.3491	0.7846	13.90						
46	7.1399	1.1946	1.0756	14.33						
85	0.3817	0.0651	0.0731	14.56						
35	5.9497	1.0506	0.7827	15.01						
49	6.2552	1.1102	0.6492	15.07						

*Filled and open boxes, respectively, indicate presence (in either homozygous or heterozogyous state) and absence of the given CYP82E genes.

Table S1 Alkaloid contents and inheritance of CYP82E genes in the F_2 plants (continued)

Plant No.	Alkaloid content in roots (µg/mg DW)			conversion (%)	Fragment Analysis*					
	nicotine	nornicotine	anatabine		*Nala CYP82E1*	*Nala CYP82E2*	*Nala CYP82E3*	*Nala CYP82E4*	*Nlan CYP82E1*	*Nlan CYP82E2*
124	0.1850	0.0336	0.0000	15.38	■	■	■		■	■
50	5.8578	1.1200	0.3857	16.05	■	■	■		■	■
105	5.9722	1.2263	0.4944	17.04	■	■	■		■	
34	0.6451	0.1366	0.1570	17.47	■	■	■		■	■
113	1.0456	0.2340	0.1203	18.29	■	■	■	■	■	■
66	3.2853	0.7451	0.6711	18.49	■	■	■		■	■
82	0.1075	0.0247	0.0000	18.67	■	■	■		■	
16	2.0177	0.4813	0.4542	19.26	■	■	■		■	■
111	2.0921	0.5048	0.2237	19.44	■	■	■		■	■
109	1.5903	0.3917	0.1121	19.76	■	■	■		■	■
94	0.9043	0.2237	0.2202	19.83	■	■	■		■	■
3	3.9420	0.9839	0.5742	19.97	■	■	■		■	■
28	0.4229	0.1086	0.0781	20.44	■	■	■		■	■
42	3.8837	1.0058	0.5126	20.57	■	■	■	■		
97	2.8613	0.7604	0.1781	21.00	■	■	■			
116	2.7772	0.7494	0.2284	21.25	■	■	■		■	■
68	3.2506	0.8848	0.4293	21.40	■	■	■	■	■	■
73	6.2027	1.7632	1.3122	22.13	■	■	■	■		
22	0.3766	0.1150	0.1279	23.40	■	■	■		■	■
71	4.8437	1.6320	0.8197	25.20	■	■	■	■	■	■
72	3.5963	1.2141	0.3869	25.24	■	■	■			
115	3.6206	1.2729	0.1717	26.01	■	■	■	■		
26	6.1778	2.2139	1.3546	26.38	■	■	■	■	■	
38	4.4242	1.6329	0.5416	26.96		■	■	■		
27	0.2647	0.0997	0.0676	27.35	■			■	■	■
104	1.3946	0.5364	0.3759	27.78	■	■	■		■	■
93	2.8324	1.1017	0.3682	28.00	■	■	■		■	■
17	2.8435	1.1745	0.1987	29.23		■	■			
102	2.9824	1.2506	0.2638	29.54	■	■	■	■	■	
92	0.4578	0.2095	0.3107	31.39	■	■	■		■	■
14	1.2663	0.5816	0.2706	31.47	■	■	■		■	■
11	0.7413	0.3470	0.1057	31.88	■	■	■			
108	1.2277	0.6225	0.1609	33.64	■	■		■		
36	2.7223	1.4438	0.9580	34.66	■	■	■	■		
86	0.2218	0.1203	0.0556	35.18	■	■	■		■	■
39	0.0659	0.0365	0.0102	35.68	■	■	■		■	■
69	6.6672	3.8257	1.8872	36.46	■	■	■	■		
122	3.8796	0.3377	0.1433	36.94	■	■	■	■		
99	0.1505	0.0967	0.0460	39.12	■	■	■	■	■	■
18	1.3739	0.9178	0.2192	40.05	■	■	■		■	
120	1.3351	0.9503	0.1999	41.58	■	■	■	■		
103	1.4365	1.0750	0.1834	42.80	■	■	■	■	■	■
40	0.0829	0.0666	0.0995	44.54	■	■	■	■	■	■
61	3.1606	2.5880	0.6938	45.02	■			■	■	
87	0.4012	0.3349	0.1263	45.49	■	■	■	■	■	■
83	0.1391	0.1397	0.0616	50.11	■	■	■			
117	1.9519	2.1505	0.2420	52.42	■	■		■		
81	0.0671	0.0757	0.0000	53.00	■	■	■			
100	0.0641	0.0810	0.0234	55.82	■	■	■	■		
101	0.3036	0.4080	0.0630	57.34	■	■	■	■		
118	0.3907	0.5693	0.0416	59.31	■	■	■	■		
80	0.4524	0.6957	0.1587	60.60	■	■	■			
70	3.0133	4.9573	0.5510	62.19	■	■	■	■		
88	0.3666	0.6648	0.2984	64.45	■	■	■	■		
89	0.0528	0.1605	0.0483	75.23		■	■			

*Filled and open boxes, respectively, indicate presence (in either homozygous or heterozygous state) and absence of the given *CYP82E* genes.

Table S2 PCR primers used in this study

Name	Sequence (from 5’ to 3’)	Comments
CYP82E-F	CCACCGAAAATCCCCGGAGGATGG	Primer set to amplify *CYP82E* subfamily genes
CYP82E-R	GCTCAGGTGCCAGGC GAGCCG	
Nala5’-1	GCCGTAAAGAAAAGCTGGACGATGG	5’-RACE gene-specific primers for four *N. alata CYP82E* genes
Nala5’-2	CGGACGGTCGTCGCCGTCGTTATCG	
Nala3’-1	CGCAAATGTTATGAAACTGCAGCG	3’-RACE gene-specific primers for four *N. alata CYP82E* genes
Nala3’-2	GG TTCTGGAAGACGATCTTGTCC	
Nlan-F1	GCTTTTATGACAGTATCACGAGAGTAACC	Inverse PCR primer set to amplify *NlanCYP82E* genes
Nlan-R1	GCGGACACAGTTGCTCTTCACATGAATTGGGG	
Nlan-F2	GCCGTAAAGAAAAGCTGGACGATTGG	Inverse PCR primer set to amplify *NlanCYP82E* genes
Nlan-R2	CGCAAATGTTATGA AACTGCAGCG	
NalaE1/2BamF	AGGATCCAAAAAAATGGTTTCCCCC	Primer set to clone *NalaCYP82E1/2* in yeast vector
NalaE1/2EcoR	TGAATTCTTAATAAAGCTCAGGTGCCAG	
NalaE3BamF	AAGGATCCAAAAAAATGGTTTCCCCCA	Primer set to clone *NalaCYP82E3* in yeast vector
NalaE3EcoR	ACGAATTCTTAATAAAGCTCAGGTGCCA	
NalaE4BamF	AGGATCCAAAAAAATGGTTCCCCC	Primer set to clone *NalaCYP82E4* in yeast vector
NalaE4EcoR	CGAATTCTTAATAAAGCTCATGTGCCA	
NlanE1BamF	CAGGATCCAAAAAAATGGTTTTTCC	Primer set to clone *NlanCYP82E1* in yeast vector
NlanE1EcoR	CCGAATTCTTAATTATAAAGCTCAGGT	
NlanE1intronF	CAACAGTTTTTAGTTTGGTCTTGGA	Primer set to remove an intron from *NlanCYP82E1*
NlanE1intronR	AAGACCAAACTAAAAACTGTTGCTT	

Table S3 PCR primers used for capillary electrophoresis-based genotyping

Name	Sequence (from 5' to 3')	Dye	Comments
1F	GGTTACTCTCGTGATACTGTCATTAAAGC	6-FAM	Primer set to amplify *NalaCYP 82E1*, *NalaCYP82E2*, *NlanCYP 82E1*, and *NlanCYPE82E2*
1R	GCAACTGTGTCCGCAGCATCCAAGACC	none	
2F	GGTTACTCTCGTGATACTGTCATTAAAGC	6-FAM	Primer set to amplify *NalaCYP 82E3*
2R	CCTAATGCTTATCAGCTACTCC	none	
3F	tgtaaaacgacggccagtCCATGATTCGAGAACTGG*	VIC	Primer set to amplify *NalaCYP 82E4*
3R	CGAAATTTTAACGGATCAAGC	none	

*Lower-case letters indicate the M13(-21) sequence.

Table S4 PCR primers used for the dCAPS assay

Target gene	Sequences of forward (F) and reverse (R) primers (from 5' to 3')	Restriction enzyme	Digested products (bp)
NalaCYP82E1	F: CGTGAAAATGATCGCGGGGAAAAATTATGACTC R: CCTTAAATGTCCTTTTCATAGCC	*Dde*I	160/30
NalaCYP82E2	F: GGTTAGAGGAACATATAAAGAAAAGAGAGCT R: CCAATTTATGTGAAGAGCAACTGTGTCC	*Sac*I	135/31
NalaCYP82E3	F: GTTAGTGGATATCACATTCCTAAAGGTAC R: CCATTGTTAAGTGTTCCACTTGCAATGC	*Kpn*I	197/29
NalaCYP82E4	F: GGCATTATTGATAAACAATCAAAAAGC R: GCGAATAGTCTAGTCCCTTTAGG	*Hind*III	213/23

Table S5 Qualitative variation of F_2 populations for nicotine converter phenotype in the roots

Cross	Total	Converter	Nonconverter	Observed ratio	Expected ratio	χ^2	*P*
F_2[a]	109	83	26	3.2 : 1	3 : 1	0.22	0.50 - 0.70

[a] F_2 = *N. alata* x *N. langsdorffii* F_1-selfed; $BC_1P_1F_1$ = *N. alata* x *N. langsdorffii* F_1 backcross to *N. langsdorffii*

LIST OF PUBLICATIONS

Pakdeechanuan, P., Shoji, T. and Hashimoto, T. (2012) Root-to-shoot translocation of alkaloids is dominantly suppressed in *Nicotiana alata*. *Plant Cell Physiol.* (in press).