Nicotine synthesis in *Nicotiana tabacum* L. induced by mechanical wounding is regulated by auxin

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Abstract

The effects of different kinds of mechanical wounding on nicotine production in tobacco plants were compared, with sand or hydroponics culture under controlled conditions. Both removal of the shoot apex and damage of the youngest unfolded leaves nos 1 and 2 by a comb-like brusher with 720 punctures caused an increase in nicotine concentration in whole plants at day 3, and reached its highest level at day 6. The nicotine concentration induced by excision of the shoot apex was much higher than that induced by leaf wounding. Both treatments also caused an increase in jasmonic acid (JA) concentration within 90 min in the shoot, followed by an increase in the roots (210 min), in which the JA concentration induced by leaf wounding was significantly higher than that induced by excision of the shoot apex. The increase in nicotine concentration occurred throughout the whole plant, especially in the shoot, while the increase in JA concentration in the shoot was restricted to the damaged tissues, and was not observed in the adjacent tissues. Removal of the lateral buds that emerged after excision of the shoot apex caused a further increase in nicotine concentrations in the plant tissues. Removal of mature leaves, however, did not cause any changes in nicotine concentration in the plant, even though the degree of wounding in this case was comparable with that occurring with apex removal. The results suggest that the nicotine production in tobacco plants was not correlated with the degree of wounding (cut-surface or punctures), but was highly dependent on the removal of apical meristems and hence on the major sources of auxin in the plant. Furthermore, immediate application of 1-naphthylacetic acid (NAA) on the cut surface after removing the shoot apex completely inhibited the increase both in nicotine in whole plants and in JA in the damaged stem segment and roots. Application of an auxin transport inhibitor around the stem directly under the shoot apex of intact plants also caused an increase in nicotine concentration in the whole plant. The results strongly suggest that auxin serves as a negative signal to regulate nicotine synthesis in roots of tobacco plants.

Key words: Auxin, jasmonic acid, mechanical damage, nicotine, tobacco (*Nicotiana tabacum* L.).

Introduction

Plants responses to herbivore attack are broadly categorized as direct and indirect defences, and tolerance. Defensive secondary metabolites are categorized by their mode of action. Proteinase inhibitors (anti-digestive proteins), polyphenol oxidases (anti-nutritive enzymes), and toxic compounds are defensive secondary metabolites (De Luca and St. Pierre, 2000; Kessler and Baldwin, 2002).

Alkaloids are toxic compounds, which poison generalist herbivores. Nicotine is an alkaloid, which only exists in tobacco plants, and accounts for ∼95% of its total alkaloid content (Baldwin, 1989; Hashimoto and Yamada, 1994). In undamaged tobacco plants, the nicotine concentration is only 0.1–1% of its dry mass, while real or simulated herbivory of tobacco leaves may cause an increase up to 1–4%, which is sufficient to deter even nicotine-adapted insects (Baldwin 1988, 1989; Ohnmeiss et al., 1997). LA Burley 21 is a genetically stable breeding line of tobacco
which is more susceptible to insect damage, probably because of its low nicotine content (Legg et al., 1970).

Nicotine is synthesized in the roots and transported in the xylem to the shoot (Tso and Jeffrey, 1956; Alworth and Rapoport, 1965). Nicotine is synthesized from the polyamine putrescine, which is produced either directly from ornithine, in a reaction catalysed by ornithine decarboxylase (ODC, EC 4.1.1.17), or indirectly from arginine, in a reaction catalysed by arginine decarboxylase (ADC, EC 4.1.1.17). As well as its role as a precursor for nicotine synthesis, putrescine also serves as a precursor in the synthesis of spermine and spermidine. The first step in nicotine biosynthesis is the conversion of putrescine to N-methylputrescine, catalysed by putrescine N-methyltransferase (PMT) (Hashimoto and Yamada, 1994; Hibi et al., 1994; Chou and Kutchan, 1998; Malmberg et al., 1998; Xu et al., 2004).

Both jasmonic acid (JA) and auxin are important for a plant’s response to wounding and pathogen attack (Hashimoto and Yamada, 1994; Hibi et al., 1994; Kutchan, 1995; Baldwin et al., 1997). Mechanical wounding of tobacco leaves can induce a 10-fold increase in JA concentration in damaged leaves within 90 min, and systemically in the roots (3.5-fold) 180 min after wounding; nicotine concentrations in leaves reached the highest level 5 d after wounding (Baldwin et al., 1997; Ohnmeiss et al., 1997). These results indicate that nicotine accumulation is a wounding response, and that JA functions as a signal molecule between the stimulus in the leaves and the response in the roots. On the other hand, many studies indicate that endogenous auxin application can inhibit a number of wound-induced responses (Mason and Mullet, 1990; Mason et al., 1992; De Wald et al., 1994). Auxin significantly reduced the methyl jasmonate (MeJA)-inducible accumulation of mRNA of ODC, S-adenosylmethionine synthase, and putrescine N-methyltransferase which are involved in nicotine synthesis (Imanishi et al., 1998a, b). In the presence of auxin, JA did not enhance the accumulation of alkaloids in cell suspension cultures of Catharanthus roseus L. (G) (Gantet et al., 1998).

Removal of the flowering head and young leaves leads to increased nicotine concentration in tobacco leaves (Hashimoto and Yamada, 1994; Hibi et al., 1994; Xi et al., 2005). However, the factors controlling the increase in alkaloid biosynthesis induced by removal of the apex are not known (Xu et al., 2004). In our field experiment, an additional increase in nicotine concentration was induced by removal of axillary buds after removal of the apex, while damage caused by routine leaf harvests did not change the leaf nicotine concentration (C Li, Q Shi, W Li, Z Zhao, F Zhang, unpublished results). It is assumed that auxin plays an important role in regulation of nicotine synthesis in roots of tobacco plants.

In this study, the effects of mechanical wounding and apex removal and the application of an auxin analogue and an auxin transport inhibitor on nicotine synthesis were investigated, in order to explain why different kinds of mechanical wounding had different effects on nicotine production. In a separate experiment, the response of JA content to mechanical wounding was also investigated, to elucidate the relationships between auxin and JA in the regulation of nicotine synthesis.

### Materials and methods

Seeds of tobacco (Nicotiana tabacum L. K 326) were germinated in a mixture of 60% (w/w) peat culture substrate, 20% (w/w) ground maize stalk, and 20% (w/w) perlite, and grown in a seedbed in a naturally lit glasshouse for 60 d. Before transfer of the tobacco seedlings into quartz sand or nutrient solution, the seedlings were washed with tap water until all substrate was removed from the roots. The following experiments were conducted.

#### Experiment 1. Effects of different mechanical wounding on nicotine concentration in tobacco plants

**Plant growth:** Tobacco seedlings were transplanted into 2.0 l pots (one plant per pot) containing quartz sand (0.25-0.50 mm in diameter). The plants were grown in a greenhouse at the China Agricultural University, Beijing. During the experimental period, the average temperature was 28–34 °C during the day and 18–26 °C at night. The plants were watered daily, initially with a quarter-strength nutrient solution containing (in mM for full strength): 1 KH2PO4, 3 KNO3, 0.25 MgSO4·7H2O, 2 Ca(NO3)2·4H2O, 0.1 Fe-EDTA, 1×10−3 MnSO4·H2O, 1×10−3 ZnSO4·H2O, 0.25×10−3 CuSO4·H2O, 0.25×10−3 (NH4)6Mo7O24·4H2O, 1×10−2 KCl, 1.25×10−2 H3BO3. After 3 d, a full-strength solution was provided. The pots were watered daily with an excess of the nutrient solution in the morning and evening, and the draining solution was removed. Different treatments were applied 90 d after sowing (~30 d after transplanting into the quartz sand).

**Treatments:** When the plants had eight unfolded leaves, including two smaller already senesced leaves, the treatments commenced. All leaves were numbered, starting with the youngest unfolded leaf, which was designated as leaf 1. There were five treatments in the experiment: (i) intact plants (control); (ii) apices were excised above the youngest unfolded leaf (removing the apex); and (iii) leaves were wounded by a plastic comb-like brush with two rows (leaf damage). Each row was 6.8 cm in length and had 18 spikes, which were equally arranged and whose diameter was 1 mm. This technique yields reproducible amounts of damage, and kills the cells at the puncture sites with minimal damage to the vasculature. Leaves 1–4 were treated, among which leaves 1 and 2 were wounded two rows on each side of and parallel to the midrib, and leaves 3 and 4 were damaged four rows on each side. The total number of punctures in leaves 1 and 2, and leaves 3 and 4 was 144 and 288, respectively; (iv) Leaves 5–8 (including two smaller senesced leaves) were excised with a razor blade (leaf excision); (v) apices were removed in the same way as in (ii), and immediately a paste consisting of 30 mM 1-naphthylacetic acid (NAA) and Tween-20 in lanolin was applied onto the decapitated stem stump (removing apex+NAA).

**Harvest procedures:** The plants were harvested 7 d after the start of the treatments. Plants were separated into roots, stems, tops (including apex and newly formed leaves after the treatments), the upper stratum of leaves 1–4 and the lower stratum of leaves 5–8. All plants parts were dried (60 °C) and weighed (dry weight). They were used for measuring nicotine concentration.
Experiment 2. Time-course of changes in nicotine concentration in tobacco plants after removal of the shoot apex

Plant growth: The transfer and plant culture were the same as in experiment 1. In this experiment, the plants were grown in a growth room with a 14 h photoperiod. The photosynthetically active radiation at the surface of the pots was 220–270 mmol m⁻² s⁻¹ provided by reflector sunlight dysposium lamps (DDF 400, Nanjing, China). The treatments started 90 d after sowing (~30 d after transfer into the quartz sand).

Treatments and harvest procedures: Four treatments were conducted in this experiment: (i) control; (ii) removing apex; (iii) in treatment (ii), lateral buds began to grow out about 7 d after excision of the shoot apex. In some of these plants, the lateral buds were removed between days 7 and 11 after removal of the apex. One lateral bud was removed each day (removing apex and lateral buds). (iv) A paste consisting of 2-chloro-9-hydroxyfluorencarboxylic acid-(9)-methylester (CFM), an auxin transport inhibitor (Beyer and Quebedeaux, 1974), and Tween in lanolin (10 mg CFM+1.0 g Tween+5.5 g lanolin) was applied annularly on the stem directly under the shoot apex of intact plants (CFM).

The control plants were harvested on the day of treatment, and at 3, 9, and 15 d after the treatments. The decapitated plants were harvested at 3, 6, 9, 12, and 15 d after removal of the shoot apex. The plants in treatment (iii) were harvested 15 d after removal of the shoot apex. The plants in treatment (iv) were harvested 3 d and 9 d after application of CFM. The harvest procedure was the same as that described for experiment 1.

Experiment 3. Time-course of changes in nicotine concentration in tobacco plants after removal of the shoot apex and leaf damage

To compare the influences of removing the shoot apex and leaf damage on nicotine production, a complementary study was performed. The plant culture conditions were the same as described in experiment 2. When the plants had six unfolded leaves, including two smaller already senesced leaves, the following treatments were commenced: (i) control; (ii) leaves 1–3 were wounded by the plastic comb-like brush with a total of 720 punctures (leaf damage). Intact plants were harvested at 0, 90, and 210 min; decapitated plants were harvested at 30, 90, 210, and 360 min; and leaves of damaged plants and NAA-treated plants were harvested at 90 and 210 min after the start of the treatments. At harvest, leaves nos 1 and 2, stem segment (internode between leaf nos 1 and 3), and root tips (3 cm in length) were harvested, and frozen immediately in liquid nitrogen, then stored at −30 °C until analysis of JA.

Analysis of JA

The methods of Baldwin et al. (1997) and Schittko et al. (2000) were used for extraction, purification and determination of JA. Frozen tissues (~0.5 g fresh weight) were homogenized in an ice-cold mixture of 70% acetone and 30% 50 mM citric acid (v/v). To the mixture, 100 ng of 9,10-dihydrojasmonic acid (Olchemim Ltd., Czech Republic) was added as an internal standard (Mueller and Broderschelm, 1994; Engelberth et al., 2003). In the pre-experiment, 9,10-dihydrojasmonic acid was not detectable in the samples (results not shown). The samples were extracted overnight at 4 °C. After centrifugation at 40 000 g at 4 °C for 20 min, the supernatant was evaporated under a N₂ stream to remove the acetone. The remaining aqueous solution was extracted with 3×3 ml of diethyl ether. The ether extracts were purified by passing them through individual aminopropyl solid-phase extraction cartridges (500 mg of sorbent; Supelco, USA). After a further washing with 7 ml of chloroform: isopropanol (3:1, v/v), JA was eluted with 6 ml of diethyl ether: acetic acid (98:2, v/v). The eluate was then evaporated to dryness under a N₂ stream and derivatized with ethereal diazomethane. After derivatization, samples were dissolved in 50 ml of ethyl acetate. A 1 ml aliquot was injected into a GC–MS (Trace 2000-Voyager, Finnigan, Thermo-Quest, USA). The methyl esters of JA and DHA were separated by gas chromatography on a DB-5 column (30 m long, 0.25 mm i.d.) with a 0.8 ml min⁻¹ He flow, and detected by MS in selective ion monitoring (electron impact of 70 eV) at m/z 224 and 226, respectively. The mass spectra of Me-JA and the methyl ester of 9,10-dihydrojasmonic acid are shown in Fig. 1.

Measurement of nicotine concentration

The nicotine concentration was analysed by the ultraviolet absorption method (Willits et al., 1950). In brief, ~0.5 g of dry sample was weighed in a dry, clean glass tube of 5 cm inner diameter, and 20 ml of distilled water and 10 ml of 30% (w/v) NaOH solution were added. The tube was placed in a distillation device and a 250 ml flat-bottomed flask was used to collect the distilled nicotine solution. Distilled water was added to make the solution up to 250 ml and then it was measured colorimetrically at 236, 259, and 282 nm using a spectrophotometer (Shimadzu UV-2201, Japan). The nicotine concentration was expressed as a percentage of the tissue dry weight.

Results

Effects of different treatments on growth of tobacco plants

Experiment 1: Removal of the shoot apex stimulated growth of the upper leaves and outgrowth of lateral buds, which was inhibited by the immediate application of the auxin analogue NAA, which appeared also to have a significantly positive effect on root growth (Table 1).
Effects of different methods of mechanical wounding and application of CFM on nicotine concentration in tobacco plants

Experiment 1: The influences of different kinds of wounding on nicotine concentrations in tobacco plants were quite different (Fig. 2). Removal of the shoot apex caused a dramatic increase in nicotine concentration, especially in the upper and lower leaves. Since the removal of the apex also led to an increase in dry weight of upper leaves, their nicotine content increased even more dramatically, while this was not true for the lower leaves. This stimulating effect on nicotine synthesis was completely suppressed by an immediate application of NAA on the cut surface. Leaf wounding also caused a slight increase in nicotine concentration, while removal of lower leaves did not cause any changes in nicotine concentration in the plant, even though the degree of wounding in this case was comparable with that occurring with apex removal.

Fig. 1. Mass spectra of Me-JA (A) and the methyl ester of 9,10-dihydrojasmonic acid (B).

Table 1. Dry weights of different organs and whole tobacco plant at harvest as affected by different methods of mechanical damage, 7 d after the start of the treatment (experiment 1)

Values in a column followed by different letters are significantly different (LSD test, P <0.05).

| Treatments            | Dry weight (g per organ per plant) |
|-----------------------|-----------------------------------|
|                       | Lateral buds | Topa | Upper leaves | Lower leaves | Stem | Roots | Whole plant |
| Control               | 8.0 a        | 6.6 b | 2.2 a        | 4.3 a         | 4.7 b | 25.8 ab |
| Removing apex         | 1.9          | 9.3 a | 2.1 a        | 2.2 c         | 4.7 b | 20.2 c |
| Leaf damage           | 8.8 a        | 7.2 b | 2.3 a        | 3.5 ab        | 4.8 ab | 26.6 a |
| Leaf excision         | 6.4 b        | 7.3 b |             | 3.6 ab        | 4.4 b | 21.7 bc |
| Removing apex+NAA     | 10.0 a       | 2.5 a |             | 3.3 b         | 5.5 a | 21.2 c |

a Including apex and newly formed leaves after the treatment.
Experiments 2 and 3: On the third day after removing the shoot apex, the nicotine concentration in whole plants, especially in the upper and lower leaves, was increased. This increase continued to the sixth day after the treatment, and then levelled off in the leaves, and decreased in stems and roots until the end of the experiment (Fig. 3). In comparison, leaf wounding only caused a slight increase in nicotine concentration in plant tissue within several days.

The nicotine concentration decreased to the control levels in all of the plant tissues at the end of the experimental period (Fig. 4).

Excision of the shoot apex stimulated outgrowth of lateral buds (Table 1). In some of the plants, the lateral buds were removed between days 7 and 11 after the first wounding, with one lateral bud per day. These repeated removals of the terminal meristems and of subsequently formed lateral meristems and the concomitant small woundings further increased nicotine concentrations in all plant parts (Fig. 5); since the plants’ dry weight was only slightly decreased by removing the small lateral buds (Table 1), the nicotine content in the whole plant also increased dramatically.

Application of CFM, an auxin transport inhibitor, around the stem directly under the shoot apex of intact plants caused an increase in nicotine concentration in the whole plant, although this treatment did not cause any wounding in the plant (Fig. 6).

Changes in JA contents in plant tissues after different treatments

JA content in leaves nos 1 and 2, stem segments (internodes between leaf nos 1 and 3), and root tips (3 cm in length) of intact plants (controls) was constantly low during the experimental period. After removal of the shoot apex, a
relatively slow (detected at 90 min) and then a strong increase in JA content in the stem segment directly under the cut surface was observed (Fig. 7). The highest JA content was reached at 210 min and this remained constant until 360 min. A delayed, slight but insignificant, increase in JA content in the roots was observed after 210 min. An immediate application of NAA after excision of the shoot apex completely inhibited the increase in JA content in all plant parts, and kept them at the control level. Leaf damage caused a relatively fast increase in JA content in the damaged leaves and then, in roots, a strong increase in JA with a delay, as detected at 210 min after the treatment. Within the shoot, the wounding or excision treatments only caused increases in JA in the wounded tissues, i.e. in the leaves after leaf wounding or in the upper stem after excision of the top, but in both cases not in other shoot tissues.

**Discussion**

In the leaf wounding experiment with different numbers of leaf punctures, a significant and positive relationship between the number of leaf punctures and nicotine accumulation was found (Ohnmeiss et al., 1997). In the present study, however, leaf wounding with a total of 432 punctures in experiment 1 (Fig. 2) and 1584 in experiment 3 (Fig. 4) caused only a slight increase in nicotine concentration. This slight increase in nicotine was apparently independent of the number of leaf punctures. In comparison, the nicotine production caused by removing the shoot apex was much more than that caused by leaf damage (Figs 2, 4),

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**Fig. 4.** Changes in the nicotine concentration of different organs of tobacco plants at different times after removing the shoot apex or leaf wounding in experiment 3. Leaves 1-3 were wounded by the plastic comb-like brush as in experiment 1. The total number of punctures in the damaged leaves was 1584.

**Fig. 5.** Changes in the nicotine concentration of different organs of tobacco plants after removing the shoot apex and excision of lateral buds after the first wounding. The lateral buds were removed one bud per day between days 7 and 11. The plants were harvested 15 d after the first wounding in experiment 2. Different letters above the columns of each organ denote significant differences at $P < 0.05$. 
although removing the apex produced only one cut surface. After removing the shoot apex, excision of lateral buds caused a further increase in nicotine concentrations in the plant tissues (Fig. 5), while excision of the lower leaves did not influence nicotine production (Fig. 2). These results demonstrate that nicotine production in tobacco plants is not correlated with the extent of the wounding (cut surface or punctures), but is highly dependent on the removal of apical meristems and hence also on the major sources of auxin in the plant (Phillips, 1975; Srivastava, 2002).

Jasmonates in plants serve key roles in gene and metabolic regulation, defence, responses to stress, reproduction, and possibly communication (Farmer and Ryan, 1992; Pena-Cortes et al., 1993; Liechti and Farmer, 2002). Damaging the stems (removing the shoot apex) or leaves increased the total amount of JA only in the damaged tissues, but not in the adjacent tissues (Fig. 7), demonstrating that JA induction was restricted to the damaged tissues (Jongsma et al., 1994; Zangerl and Berenbaum, 1995; Ohnmeiss et al., 1997). However, the increase in nicotine concentration was not only localized in the damaged tissue, but occurred in the whole plant. This is one of the typical responses of plants to avoid further herbivore attack (Hashimoto and Yamada, 1994; De Luca and St Pierre, 2000; Kessler and Baldwin, 2002). Since the damage occurred in the shoot and nicotine is synthesized in the roots and transported in the xylem to the shoot (Tso and Jeffrey, 1956; Alworth and Rapoport, 1965), a signal is necessary to connect the stimulus in the shoot and the response in the roots. Several studies provide evidence that JA regulates nicotine synthesis in the roots (Baldwin et al., 1994, 1997; Gantet et al., 1998; Imanishi et al., 1998a). The result that girdling above, but not girdling below the position of the damage inhibited nicotine synthesis implies an involvement of phloem signal transport in the regulation of stimulated synthesis of nicotine in roots (Baldwin, 1989). The present results show that leaf wounding led to a substantial increase in young leaf JA within 90 min, and subsequently (detected at 210 min) to
a significant increase in root JA (Fig. 7), which led to an increase in nicotine concentration (Fig. 4). These results appear to indicate that JA is translocated from the leaves to the root (Zhang and Baldwin 1997). On the other hand, apex excision also increased the JA concentration in the adjacent upper stem segment within 90 min. However, this increase in JA did not lead to a subsequent, substantial increase in the root JA (in comparison with the control and 30 min after apex excision), although this treatment leads to major increases in nicotine concentration in the whole plant. The nicotine concentration induced by excision of the shoot apex was significantly higher than that induced by leaf wounding. The results appear to show clearly that, at least according to the presented data, JA was not responsible for the massive increase in nicotine synthesis after removal of apical meristems.

Auxin is mainly synthesized in the apex and young leaves, and transported down the stem of the plant at the vegetative stage (Phillips, 1975; Srivastava, 2002). The results in the present study demonstrated that removal of the apical meristems—either only the shoot apex or subsequently also the lateral buds—with their physiological functions, i.e. amongst others being sources of auxin, has a much stronger effect on nicotine synthesis in the root than a much more severe wounding of the young leaves. This was supported by the finding that the effect of apex removal on nicotine synthesis could be (i) mimicked by application of an auxin transport inhibitor and (ii) reversed by replacement of the apical tissues by an auxin analogue. On the other hand, removal of the mature leaves could not stimulate nicotine synthesis, since the old leaves are not capable of synthesizing auxin (Phillips, 1975; Srivastava, 2002). It has been found that nicotine synthesis was stimulated by reduction of the medium auxin concentration in callus tissue originating from N. tabacum cv. Sam. (Feth et al., 1986), and exogenous indole-3-acetic acid (IAA) or NAA suppressed the nicotine response to wounding when it was applied directly to the wounded leaf (Baldwin et al., 1997; Baldwin, 1989). Wounding N. tabacum leaves caused a decline in endogenous IAA in the same leaves (Tomburg and Li, 1991). The reduced supply of auxin may result in activation or derepression of genes involved in nicotine synthesis, such as ODC and PMT (Hibi et al., 1994; Imanishi et al., 1998a). In conclusion, the results strongly suggest that auxin serves as a negative signal to regulate nicotine synthesis in the roots of tobacco plants.

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