The Jasmonate-Induced Expression of the *Nicotiana tabacum* Leaf Lectin

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Previous experiments with tobacco (*Nicotiana tabacum* L. cv Samsun NN) plants revealed that jasmonic acid methyl ester (JAME) induces the expression of a cytoplasmic/nuclear lectin in leaf cells and provided the first evidence that jasmonates affect the expression of carbohydrate-binding proteins in plant cells. To corroborate the induced accumulation of relatively large amounts of a cytoplasmic/nuclear lectin, a detailed study was performed on the induction of the lectin in both intact tobacco plants and excised leaves. Experiments with different stress factors demonstrated that the lectin is exclusively induced by exogenously applied jasmonic acid and JAME, and to a lesser extent by insect herbivory. The lectin concentration depends on leaf age and the position of the tissue in the leaf. JAME acts systemically in intact plants but very locally in excised leaves. Kinetic analyses indicated that the lectin is synthesized within 12 h exposure time to JAME, reaching a maximum after 60 h. After removal of JAME, the lectin progressively disappears from the leaf tissue. The JAME-induced accumulation of an abundant nuclear/cytoplasmic lectin is discussed in view of the possible role of this lectin in the plant.

**Keywords:** Inducible protein — Jasmonate — Lectin — *Nicotiana tabacum* — *Spodoptera littoralis* — Tobacco.

Abbreviations: AOC, allene oxide cyclase; BA, 6-benzylaminopurine; DMSO, dimethylsulfoxide; GA3, gibberellic acid; JA, jasmonic acid; JAME, jasmonic acid methyl ester; 12-OH-JA, tuberonic acid; Nictaba, *Nicotiana tabacum* agglutinin; OPDA, 12-oxo-phytodienoic acid; RT–PCR, reverse transcription–PCR; SA, salicylic acid.

**Introduction**

Many plants including important food crops such as wheat, potato, tomato and bean contain carbohydrate-binding proteins commonly referred to as ‘lectins’, ‘agglutinins’ or ‘hemagglutinins’ (Van Damme et al. 1998, Van Damme et al. 2007). Plant lectins represent a very diverse and heterogeneous group of plant proteins that contain at least one non-catalytic domain that binds reversibly to specific mono- or oligosaccharides. The characterization of an extensive number of different plant lectins revealed, however, the existence of only a limited number of carbohydrate-binding motifs (Peumans et al. 2000). Most lectins exhibit a sugar specificity directed against complex N- and O-glycans that are absent from plant cells, but present on the surface of microorganisms or on the epithelial cells along the intestinal tract of phytophagous invertebrates and/or herbivorous animals. Together with the high expression levels (generally 0.1–10% of the total protein) and preferential accumulation in storage tissues, it is believed that these so-called ‘classical’ plant lectins do not fulfill an endogenous role in the plant, but preferably function as storage proteins and, whenever appropriate, can also act as defense proteins (Van Damme et al. 2007). It has already been shown that some plant lectins possess cytotoxic, fungitoxic, anti-insect and anti-nematode properties in vitro or in vivo, and some lectins are toxic to higher animals (Van Damme et al. 1998, Van Damme et al. 2007).

The role of lectins in plant defense against foreign attack is in marked contrast to the function of animal lectins because most of these lectins are believed to recognize and bind ‘endogenous’ receptors and, accordingly, are involved in recognition mechanisms within the organism itself (Kilpatrick 2002, Sharon and Lis 2004). However, the recent finding of several stress-inducible plant lectins opens up new perspectives for an endogenous role for a new class of plant lectins (Van Damme et al. 2004a, Van Damme et al. 2004b). Most of these ‘inducible’ lectins are expressed at very low levels only after exposure of the plant to specific biotic/abiotic stimuli, such as salt stress, drought, light, heat or cold shock, wounding or treatment with ABA, jasmonic acid (JA) and gibberellins (Van Damme et al. 2007). Unlike the classical lectins which are present in vacuoles, this new class of plant lectins is located exclusively in the cytoplasm and the nucleus. Based on these observations, the concept was developed that lectin-mediated protein–carbohydrate interactions in the cytoplasm and the nucleus play an important role in...
the stress physiology of the plant cell (Van Damme et al. 2004a, Van Damme et al. 2004b).

Although it is widely known that plant tissues treated with jasmonates or exposed to chemicals that induce jasmonate accumulation in planta synthesize high levels of so-called jasmonate-induced proteins (Wasternack and Hause 2002, Wasternack 2006), the first jasmonate-induced lectin was only reported in 2002, when Chen et al. first described the accumulation of a chitin-binding lectin in tobacco (Nicotiana tabacum L. cv Samsun NN) leaves, also called Nicotiana tabacum agglutinin or Nictaba. Lectin activity cannot be detected in untreated tobacco leaves but accumulates in leaves treated with jasmonic acid methyl ester (JAME). No lectin expression was detected in vascular tissues, stems, flowers and roots (Chen et al. 2002). The leaf lectin was purified and characterized as a dimeric protein composed of 19 kDa subunits that are not glycosylated. Recently it was shown that Nictaba not only recognizes GlcNAc oligomers but also strongly interacts with high-mannose and more complex N-glycans (Lannoo et al. 2006b). Using immunocytochemistry with an antibody specifically directed against the lectin, the location of Nictaba in the nucleus and to a lesser extent in the cytoplasm was shown in JAME-treated leaves (Chen et al. 2002). Meanwhile these results were confirmed by confocal microscopy of the expression of an enhanced green fluorescent protein (EGFP)-Nictaba fusion construct in tobacco cells. These microscopic analyses further revealed an accumulation of the lectin at the nuclear rim (Lannoo et al. 2006b).

Although the carbohydrate-binding activity and specificity of Nictaba are well understood, the question remains why tobacco leaves accumulate relatively large amounts of a cytoplasmic/nuclear lectin in response to jasmonates. To obtain first insights into the physiological role of Nictaba, a detailed study was made of the induction of this new type of lectin in both intact tobacco plants and excised leaves. The different factors that can induce lectin activity as well as the timing of lectin accumulation in plant tissues are discussed.

**Results**

**Jasmonic acid and its methyl ester are the key chemical inducers of the tobacco lectin**

To identify possible inducing agents other than jasmonates, excised leaves of tobacco (N. tabacum cv Samsun NN) plants were subjected to a series of treatments with chemicals or abiotic factors and checked for the induction of lectin activity using agglutination assays. Of all plant hormones and hormone-releasing compounds tested [JA, 12-OH-JA, JAME, gibberellic acid (GA₃), salicylic acid (SA), IAA, ABA, 6-benzylaminopurine (BA) and ethephon], only JAME and JA induced the synthesis of lectin in detached leaves of tobacco plants, the optimal concentrations being 50–100 μM for JAME and 100–150 μM for JA (Fig. 1) (data not shown for most plant hormones). Lectin accumulation could be induced by floating leaves on a solution containing JA or JAME, as well as by treatment of plants with JAME through the gas phase. Combinatorial treatment of the leaves with different plant hormones either followed or preceded by a JAME treatment did not reveal any noticeable synergistic or inhibitory effect between JAME and other plant hormones. Repeated mechanical wounding by different techniques failed to induce any detectable lectin activity. The same holds true for salt stress, heat and cold shock, and irradiation with UV light (data not shown).

Though induction of lectin activity was observed with detached leaves of plants grown in vitro as well as greenhouse-grown plants, excised leaves cut from plants grown in vitro accumulated less lectin compared with those cut from greenhouse-grown plants of identical age (250 μg g⁻¹ FW and 3 mg g⁻¹ FW, respectively). Estimations of the lectin content of all individual leaves of a JAME-treated plant revealed that the rapidly expanding leaves accumulate more lectin (up to 500 μg lectin g⁻¹ FW) than both older and younger leaves (expressing 100 and 200 μg lectin g⁻¹ FW, respectively), indicating that the responsiveness of the leaves changes as a function of age. Therefore, leaves of a comparable age and developmental stage were used for all comparative analyses.

**Kinetics of the JAME-induced Nictaba accumulation in excised tobacco leaves**

To follow the kinetics of lectin accumulation in detached leaves, leaves cut from 12-week-old
greenhouse-grown plants were floated on a 50 μM JAME solution for different time periods and subsequently transferred onto water until the end of the experiment (72 h). Then, the leaves were extracted and their lectin content determined. As shown in Fig. 2, treatment with JAME for at least 12 h is required to induce a detectable level of Nictaba. However, exposure to JAME for 48–60 h is required to reach the maximum level of JAME-induced Nictaba.

Though indicative for the requirement of a relatively long exposure time, the results do not prove or disprove the need for a continuous exposure to JAME. To address this question, a similar set of leaves was subjected to a daily induction regime where they were floated on a 50 μM JAME solution for a given time followed by incubation on water for the rest of the day. This regime was followed for three consecutive days, after which the leaves were extracted and the lectin content determined (inset in Fig. 2). A short JAME treatment of 2 h for three consecutive days already resulted in the induction of an amount of lectin comparable with that observed after a continuous exposure to JAME for 24 h. A daily JAME treatment for 6 h interrupted by 18 h flotation on water for three consecutive days yielded a final lectin concentration equal to that of leaves that were continuously exposed to JAME for approximately 40 h. These findings indicate that the induction of Nictaba by exogenous JAME does not require a continuous exposure but can also be achieved by intermittent daily JAME applications.

**In detached leaves Nictaba expression is restricted to the site of JAME application**

To check whether Nictaba accumulates uniformly over the whole leaf area, leaves were cut from 16-week-old greenhouse-grown plants and transferred onto a 50 μM JAME solution. After incubation for 3 d the leaves were divided (along the longitudinal axis) in 1 cm slices. Extraction and subsequent determination of the lectin content revealed that the lectin amount was highest in the slices originating from the middle part of the leaf (reaching levels up to 30 mg g⁻¹ FW) and decreased towards both the proximal and distal end (Fig. 3A). At the very tip, the lectin amount was approximately 10-fold lower than in the middle of the leaf. At the proximal end, the slice consisted almost exclusively of the petiole or mid rib, and lectin activity was barely detectable. Similar results were obtained with leaves from whole plants treated with JAME through the gas phase for 4 d, indicating that the responsiveness of the parenchyma cells is for a great part determined by their position in the leaf. Experiments in which only part of a detached leaf (top, middle or bottom part) was floated on JAME (and the remainder of the leaf floated on water) revealed that lectin accumulation was detectable only in that part of the leaf that was in direct contact with the JAME solution (Fig. 3B–D). The tissues that were immersed in water did not accumulate detectable amounts of lectin, suggesting that in detached leaves JAME acts exclusively at the site of application.

**Nictaba expression is systemically induced in tobacco plants**

To address whether JAME acts systemically or locally in a plant, a single leaf of a 4-week-old greenhouse-grown tobacco plant, still attached to the plant, was placed in a closed Petri dish filled with a 50 μM JAME solution. After incubation for 3 d, extracts were made of all individual leaves and assayed for lectin activity. As could be expected, the treated leaf accumulated a high level of Nictaba. Lectin activity was also detected in all other leaves, suggesting transport of a signal from the treated leaf to the rest of the plant. However, the lectin content depends on the position of the leaf relative to that of the leaf treated with the JAME solution (Fig. 4). The basal leaves contained lectin, but the level of activity was very low. In the apical leaves, the lectin content was much higher, except in the leaf just above the treated leaf. The lectin content of the second and third apical leaf was almost as high as that of the treated leaf. Towards the top, the amount of Nictaba progressively decreased. Though a minimal response is observed in the basal direction, the predominant response appeared in the apical direction. The low response of the first apical leaf might be due to its position opposite to the treated leaf. Apical transport of an inducer starting
from the base of the treated leaf is at the height of the first apical leaf mainly confined to the vascular bundles at the opposite site so that only a very weak signal can be transmitted in this first upper leaf.

Fate of JAME-induced accumulation of Nictaba

The availability of an in planta system consisting of whole plants induced through the gas phase and a system of excised leaves floating on a JAME solution offered the opportunity to compare the amount of lectin after removal of its inducer in leaves that remain fully functional on the plant and in leaves that are irreversibly directed towards aging and functional disintegration after being detached from the plant. To follow the fate of Nictaba, 14-week-old plants with large-sized leaves (approximately 40 cm) were treated with JAME in the gas phase whereas detached leaves were floated on a solution of JAME. Samples were taken regularly from the central part of single leaves of comparable age and position on the tobacco plants (to minimize the variability of the lectin content due to the position of the leaf on the plant and the position of the

![Graph](https://example.com/graph1)

**Fig. 3** Distribution of Nictaba in detached tobacco leaves treated with 50 μM JAME for 4 d. (A–D) Application of JAME to the whole leaf (A), the middle part (B), the tip (C) and the basal part (D) of the leaf, respectively. The leaf area treated with the JAME solution is shaded gray. After incubation, leaves were cut in 1 cm slices and individually extracted and assayed for lectin activity. Slices are numbered from the base to the top of the leaf.

![Graph](https://example.com/graph2)

**Fig. 4** Systemic induction of Nictaba in different leaves of a 4-week-old greenhouse-grown tobacco plant after floating of a single fully expanded leaf (while still attached to the plant) on 50 μM JAME for 3 d. At the end of the induction period, all leaves were individually extracted and their lectin amount determined. The leaf treated with JAME is numbered 0 and shaded gray. Apical leaves are numbered 1–8 (from leaf 0 to the top); distal leaves are numbered −1 to −7 (from leaf 0 to the base).
tissue within the leaf). Since mechanical wounding does not induce Nictaba it is unlikely that the repeated sampling from the same leaf interferes with the fate of the lectin in the rest of the leaf. Analyses of detached leaves that were floated on a JAME solution for 3 d, extensively washed with water and subsequently incubated on daily refreshed water for up to 3 weeks demonstrated that the amount of lectin decreased rapidly with a half-life of approximately 5 d. After 20 d, lectin levels were barely detectable, but at that time the leaves already started to decay (Fig. 5A). Using the in planta system, in which plants were treated with JAME in the gas phase for four consecutive days and samples were taken regularly over a period of 50 d after treatment, quantitative analyses showed that the induced Nictaba amount remained unaffected during the first 10 d after JAME removal, but then decreased progressively. After 20 d, the lectin content was diminished to 25% of the initial amount, and fell below the level of detection after approximately 6 weeks (Fig. 5B). In conclusion, the decrease in the amount of lectin in whole plants after removal of the inducer occurs much more slowly with a half-life of approximately 10 d compared with the experiment with detached leaves.

**Insect damage induces lectin expression**

To test the effect of insect herbivory, 16-week-old greenhouse-grown tobacco plants were infested with larvae of the cotton leafworm (Spodoptera littoralis) whereby a single larva was allowed to feed on one leaf for 12 h. After feeding, larvae were removed and lectin contents were measured immediately in both the infested leaf and two non-infested upper leaves and one non-infested lower leaf (= systemic leaves). Agglutination assays with leaf extracts from the plant subjected to insect herbivory did not show lectin activity in the wounded leaf or in the systemic leaves. However, transcription of the *Nictaba* gene(s) could be demonstrated in all treated tobacco leaves after insect damage using reverse transcription-PCR (RT-PCR) for amplification of RNA for Nictaba (Fig. 6A, 608 bp band). In control leaves and leaves positioned near the treated leaves, no RNA for Nictaba could be detected in the first round of PCR. When a nested PCR was performed, all non-treated tobacco leaves also showed a faint signal for Nictaba expression (Fig. 6A, 498 bp band), suggesting the presence of low RNA levels for Nictaba in these leaves. Using this nested PCR, Nictaba RNA was also detected in the control leaves, suggesting the presence of a basal transcription activity of the *Nictaba* gene(s) which, however, does not result in detectable expression of the protein when assayed by agglutination assays or Western blot.

Insect herbivory on tobacco leaves also clearly induced expression of a gene encoding allene oxide cyclase (AOC), an important enzyme in the biosynthesis pathway of jasmonates. RT-PCR revealed the presence of RNA encoding *NtAOC* in the treated leaves as well as in the first leaf above the treated one (Fig. 6A, 776 bp band), implicating the systemic induction of this enzyme as reported previously (Stenzel et al. 2003b). Using a nested PCR, *NtAOC* RNA was also detected in the second leaf above the treated leaf (Fig. 6A, 384 bp band).

Quantification of JA levels in the leaf samples indicated that insect damage enhanced the endogenous levels of the JA precursor 12-oxo-phytodienoic acid (OPDA), JA and the JA metabolite 12-OH-JA (also known as tuberonic acid) in treated leaves but not in systemic leaves (Table 1).
Similar observations were made when a single leaf was floated on 50 μM JAME, while still attached to the plant. Remarkably, the amount of 12-OH-JA was far more elevated by insect damage than the amounts of OPDA and JA, and was dramatically increased upon JAME treatment.

In a second experiment, a single leaf of a tobacco plant was infested with larvae of the cotton leafworm for different time periods ranging from 3 h up to 24 h. Lectin activity was quantified 48 h after the start of the experiment, allowing time for protein synthesis. As shown in Fig. 6B, insect herbivory clearly induced lectin activity in the wounded leaves. Maximal levels of lectin (300 μg Nictaba g−1 FW) were reached when caterpillars were allowed to eat for approximately 15 h. Longer feeding times did not further increase the lectin levels induced in the infested leaves.

Table 1  Determination of the concentrations of oxo-phytodienoic acid (OPDA), JA and 12-OH-JA in N. tabacum cv Samsun NN leaves after S. littoralis herbivory and JAME treatment

<table>
<thead>
<tr>
<th>Insect herbivory</th>
<th>JAME treatment</th>
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<tr>
<td></td>
<td>[OPDA] (pmol g−1)</td>
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<td></td>
<td></td>
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<tr>
<td>Control leaf</td>
<td>103.7 ± 15.1</td>
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<tr>
<td>Leaf 1–</td>
<td>80.7 ± 12.9</td>
</tr>
<tr>
<td>Treated leaf</td>
<td>113.7 ± 2.5</td>
</tr>
<tr>
<td>Leaf 1+</td>
<td>85.0 ± 3.6</td>
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<tr>
<td>Leaf 2+</td>
<td>80.7 ± 3.18</td>
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<td></td>
<td>84.7 ± 23.0</td>
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<td>94.0 ± 5.6</td>
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<td></td>
<td>102.0 ± 3.6</td>
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<td>62.3 ± 36.7</td>
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<td>86.7 ± 10.7</td>
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Samples were taken from the treated leaf as well as the first leaf below the treated leaf (leaf 1–) and the two leaves above the treated leaf (leaf 1+, leaf 2+ respectively). A leaf taken before the treatment served as a control. Each value is the mean of three independent experiments.

a A single L4 larva of S. littoralis was allowed to feed on one leaf of a tobacco plant for 12 h. Afterwards all leaves were collected and analyzed immediately.

b One leaf of a tobacco plant was floated on 50 μM JAME in a closed container while still attached to the plant. After 12 h all leaves of the plant were collected and analyzed immediately.
Discussion

Jasmonates are important signaling molecules that function in (i) developmental processes; (ii) regulation of the plant’s metabolism; and (iii) defense responses against pathogens and insects/herbivores by altering the expression of defense proteins, enzymes of amino acid and secondary metabolism, and vegetative storage proteins (for reviews, see Creelman and Mullet 1997, Kessler and Baldwin 2002, Howe 2004, Devoto and Turner 2005, Wasternack 2006). Earlier reports have shown that jasmonates do not provoke the same or a similar set of responses in all plants (reviewed in Wasternack et al. 1996). Previous studies analyzing jasmonate responses in tobacco reported the up-regulation of enzymes directly involved in the oxylipin pathway (i.e. the synthesis pathway for jasmonates) such as hydroperoxide lyase and lipooxygenase, the up-regulation of acid phosphatase, the production of several volatile C6 compounds, and a reduction of the overall protein content after application of signaling compounds, JA or JAME (Avdiushko et al. 1995, Kenton et al. 1999b).

Here we report the accumulation of a carbohydrate-binding protein from N. tabacum as a response to exogenous application of different stresses. From all plant hormones and chemical compounds tested, only JA and JAME induced Nictaba accumulation in tobacco leaves. Treatment with other plant hormones such as SA or ethylene did not visibly change the jasmonate-induced lectin content. An antagonistic effect of SA on JA/ethylene signaling in plant defense has been reported on several occasions (Peña-Cortés et al. 1993, Doares et al. 1995, Niki et al. 1998, Takahashi et al. 2004), whereas only some examples of a synergistic effect have been published (Campbell et al. 2003, Mur et al. 2006). Mur et al. (2006) claim that the outcome of SA/JA signaling in plant defense pathways is highly dependent on the relative concentrations of each hormone. The absence of any antagonistic or synergistic action of other plant hormones on the level of Nictaba induction could be explained by the fact that lectin induction by JA is a very late response (after several hours or even days, see Fig. 2). In tomato it was shown that transcripts of so-called late response genes, encoding proteinase inhibitors and other defense-related proteins, start to accumulate locally and systemically about 2 h after wounding and reach maximum levels 8–12 h after wounding (Howe 2004). Genes exhibiting more rapid and transient expression (i.e. within minutes after stress application) comprise early wound response genes. This last group of genes encodes components of the JA-mediated wound response pathway including (pro)systemin and the JA biosynthetic genes such as LOX, AOS, AOC and OPR3 (Ryan 2000, Wasternack and Hause 2002, Howe 2004, Schilmiller and Howe 2005). All previously reported examples of JA- and wound-induced gene expression in tobacco are clearly late responses (appearing within a few hours) and correspond to the wound-induced transient increase in JA content within 1 h (Wasternack et al. 1996). According to Fig. 2, the JA-induced accumulation of Nictaba, however, appears very late, with maximal lectin levels after 2 d. Apparently, the inductor needs to be present at sufficiently high concentrations for a certain time period in order for the lectin expression to be switched on. This may explain why no treatment other than JA or JAME results in Nictaba accumulation. Some of the treatments tested, such as mechanical wounding, are known to provoke a transient rise of jasmonates within 1 h of treatment to levels in the nanomolar range per gram FW (Kenton et al. 1999a, Mur et al. 2006). Obviously, this level of JA is insufficient for Nictaba accumulation.

The highest lectin levels as a response to JA and JAME treatment were found in the expanding tobacco leaves, suggesting a developmental and/or defense-related lectin induction. Consistent with this hypothesis is our observation that Nictaba is not only induced by jasmonates but also after caterpillar attack of larvae of the (polyphagous) cotton leafworm S. littoralis. Though the amount of lectin induced in damaged leaves after insect feeding is lower than in tobacco leaves treated with JAME, insect herbivory definitely induces the synthesis of Nictaba. It was shown that exposure of tobacco leaves to insect herbivory for 3 h resulted in detectable levels of lectin activity, whereas approximately 15 h herbivory was needed to achieve maximal expression levels. It should be noted that insect feeding generates a lower internal JA concentration in the treated leaf compared with floating of a leaf on a JA/JAME solution. This lower JA concentration could be responsible for the lower expression levels of Nictaba.

The JA pathway is known to play a central role in the plant’s response to insect herbivory. Chewing insects such as caterpillars cause extensive cellular disruption, which plays an important role in plant perception of these herbivores. In the last decade it was shown that insect herbivory has an additional effect on the plant’s response compared with mechanical wounding of the plant tissue (Reymond et al. 2000, Walling 2000). Importantly, insect feeding causes a larger increase in JA than wounding alone. In addition, insect oral secretions modulate plant transcript profiles and induce expression of a distinct set of genes as well as synthesis and release of different volatiles (Korth and Dixon 1997, Kessler and Baldwin 2002, Ferry et al. 2004, Lawrence and Novak 2004). Possibly some compounds in the insect saliva are also required for the induction of lectin synthesis.

Another possible explanation is that—as is suggested by the experiments shown in Figs. 2 and 6—the exposure time to JAME must surpass a threshold value before lectin
Jasmonate-induced expression of tobacco lectin

Mithöfer et al. (2005). Similar to Nictaba, the expression of the hevein-like protein of Arabidopsis was also shown to be induced by insect feeding but not by mechanical wounding (Reymond et al. 2000).

Jasmonate-dependent signaling may also be influenced by metabolic transformation of jasmonates. As shown in Table 1, a remarkable portion of JAME is cleaved, presumably by an esterase (Stuhlfelder et al. 2004), whereas JAME might be formed upon JA treatment due to the presence of a JA-specific methyltransferase (Seo et al. 2001). We found an equilibrium of 80% JA and 20% JAME in plants such as tobacco and tomato (O.M., unpublished results). The need for a threshold level of JAME for Nictaba induction is supported by the fact that in detached leaves the lectin accumulates exclusively at the site of JAME application (Fig. 3). In contrast, a systemic induction of Nictaba was observed in whole plants (Fig. 4). In recent years, several reports have shown evidence that jasmonates are transported in the plant (Baldwin et al. 1997, Zhang and Baldwin 1997, Schilmiller and Howe 2005, Thorpe et al. 2007). Recent experiments have clearly shown that labeled JAME is taken up in N. tabacum leaves and moves in both phloem and xylem pathways (Thorpe et al. 2007). The lower lectin content in the first leaf above the treated leaf can result from the lack of vascular connectivity between leaves in opposite orthostichies (Orians et al. 2000, Orians, 2005). However, Thorpe et al. (2007) have shown that the volatile JAME can also move between non-orthostichous vascular pathways. They could rule out the possibility that the JAME observed in the systemic leaves is the result of volatile release by other parts of the plant. Taking all these results together, it seems more likely that systemic induction of Nictaba in tobacco leaves is mediated by a signal traveling within the plant rather than the volatile JAME acting through the atmosphere.

Unlike JA and JAME, the JA derivative 12-OH-JA fails to induce Nictaba. This is in agreement with previous observations that not all activities associated with JA are shared by its metabolites. For example, both JA and 12-OH-JA induce tuber formation in potato (Koda et al. 1991), but only JA and its amino acid conjugates and not 12-OH-JA induce the expression of jasmonate-induced proteins JIP-23 and JIP-6 (Miersch et al. 1999). We have shown that although the levels of both JA and 12-OH-JA accumulate 3- to 5-fold within the leaf attacked by S. littoralis (Table 1), only JA induced expression of Nictaba. As expected, JAME treatment led to a high JA content presumably by the activity of an esterase (Stuhlfelder et al. 2004). The dramatic increase in 12-OH-JA levels following JAME treatment, but lack of lectin accumulation following 12-OH-JA treatment (Fig. 1), is consistent with some data recently obtained with tomato. In tomato, a JA-dependent 12-OH-JA formation was observed, which blocked JA-induced gene expression (O. Miersch et al., in preparation). Obviously, the formation of 12-OH-JA is a mechanism to halt JA signaling.

Nictaba is not the first lectin induced by jasmonates. Other jasmonate-inducible lectins have been reported such as the myrosinase-binding proteins from Arabidopsis and Brassica species (Geshi and Brandt, 1998), the mannose-specific lectin from Helianthus tuberosus callus (Nakagawa et al. 2003) and some jacalin-related lectins in Poaceae species (Van Damme et al. 2004c). However, the jasmonate-induced accumulation of Nictaba clearly differs from that of the jacalin-related lectins. The presumed jasmonate-induced Brassica napus myrosinase-binding proteins are also formed (albeit at a lower level) in the absence of jasmonate (Geshi and Brandt 1998), whereas mannose-binding jacalins in cereals such as wheat, barley, rice and maize are not exclusively induced by jasmonates but also by other stress factors (e.g. salt stress, intense light, ABA) (Van Damme et al. 2004c).

At present we can only speculate about the physiological role of the tobacco lectin in the plant. In view of the localization pattern showing an accumulation of Nictaba at the nuclear rim, it has been hypothesized that Nictaba might play a role in transport of molecules in and out of the nucleus. It can be envisaged that Nictaba is able to interact with, for example, glycosylated nucleoporins located in the nuclear envelope. Evidence for the interaction of Nictaba with nuclear tobacco proteins was recently obtained from Far Western blots which clearly demonstrated that Nictaba reacts in a GlcNAc oligomer-inhibitable manner with numerous proteins present in a crude extract from purified nuclei (Lannoo et al. 2006b). This, taken together with the nucleocytoplasmic location and the induction by jasmonate, strongly argues for a specific role for Nictaba in jasmonate-inducible or jasmonate-dependent physiological processes (Chen et al. 2002, Van Damme et al. 2004a). In order to obtain better insight into the physiological role of these lectins, future experiments should aim at the characterization of the interactors for these lectins in the plant. In view of the induced lectin accumulation after insect attack, the insecticidal properties of the lectin as well as the lectin receptors in the insect body will also be investigated in more detail. In this respect it is interesting to note that several herbivore- and JA-inducible proteins occur in the midgut of herbivores and can act in a synergistic manner (Chen et al. 2005, Chen et al. 2007).
Materials and Methods

Plant material and growth conditions

Tobacco (N. tabacum L. cv Samsun NN) seeds were purchased from Lehle Seeds (Round Rock, TX, USA). Prior to use, seeds were surface sterilized with 70% (v/v) ethanol for 2 min, 7% (v/v) NaOCl for 10 min, and extensively washed with sterile water. To establish an in vitro culture, seeds were germinated on solid Murashige and Skoog (MS) medium containing 4.3 g l⁻¹ MS micro- and macronutrients containing vitamins (Duchefa, Haarlem, The Netherlands), 30 g l⁻¹ sucrose, pH 5.7 (adjusted with 0.5 M NaOH) and 8 g l⁻¹ plant agar (Duchefa). After germination, plants were transferred to MS medium containing 2.15 g l⁻¹ micro- and macronutrients containing vitamins. Plants were kept in a growth chamber at 25°C, 70% relative humidity and a 16 h photoperiod, and propagated through cuttings every 6–7 weeks. For the production of in vivo grown plants, seeds were germinated in Petri dishes filled with pot soil. After the appearance of the cotyledons and first leaves, plantlets were transferred to bigger pots filled with pot soil and kept in the greenhouse until flowering.

Plant hormones, hormone-releasing and abiotic compounds

JA, GA₃, SA, IAA (all from Duchefa), JAME (Sigma, St Louis, MO, USA); 12-OH-JA (a gift of T. Yoshihara, Sapporo, Japan) and ABA (Acros Organics, Geel, Belgium) were first dissolved in a small volume of absolute ethanol and subsequently diluted with water. Ethephon (from Acros Organics) was dissolved in 100% dimethylsulfoxide (DMSO) and diluted with water to the desired concentrations. BA (Acros Organics) was dissolved in 100% DMSO and subsequently diluted with water to the desired concentrations. BA (Acros Organics), 12-OH-JA (a gift of T. Yoshihara, Sapporo, Japan), and ethylene (from Acros Organics) was dissolved in 100% DMSO and further pre-purified as described by Stenzel et al. (2003a). For HPLC separation, fractions at retention time (Rt) 9.75–10.75 min (12-OAc-JA), 13–14.5 min (JA) and 12.32 min; 12-[2H₃]OAc-JA, 17.16 min; 12-[2H₅]OPDA, 21.98 min. For wounded experiments, leaves were either rubbed with carborundum powder, cut in pieces or clipped. Alternatively, one leaf of a tobacco plant was subjected to insect herbivory for different time periods (3–24 h). The leaf material was collected 48 h after the start of the experiment and analyzed for lectin activity as described above.

Quantification of endogenous jasmonate concentration

Fresh plant material (500 mg) was homogenized with 10 ml of methanol and 100 ng each of [²H₆]JA (Miersch 1991), [²H₅]OPDA and 12-[²H₃]OAc-JA as internal standards. The homogenate was filtered under vacuum on a column containing cellulose. The eluent of acetic acid anhydride at 20°C, evaporated and acetylated with 200 μl of pyridine and 100 μl of acetic anhydride at 20°C. The sample was derivatized with pentafluorobenzyl esters were eluted from SiOH cartridges with hexane:ether (1:1, v/v) and measured by gas chromatography–mass spectrometry (GC-MS) using the following conditions: 100°C, negative chemical ionization, ionization gas NH₃, ion source temperature 140°C, column Rtx-5w/Integra Guard (Restek, Germany), 5 m inert pre-column connected with a 15 m x 0.25 mm column, 0.25 μm film thickness, cross-bond 5% diphenyl–95% dimethyl polysiloxane, injection temperature 220°C, interface temperature 250°C, helium 1 ml min⁻¹, splitless injection and a column temperature program of 1 min 60°C, 25°C min⁻¹ to 180°C, 5°C min⁻¹ to 270°C, 10°C min⁻¹ to 300°C. The Rt of pentafluorobenzyl esters were: [²H₆]JA, 11.80 min; [²H₅]OPDA, 12.24 min; [²H₃]OAc-JA, 17.16 min; 12-[²H₅]OPDA, 21.93 min; trans-OPDA, 21.35 min; cis-OPDA, 21.98 min. The fragmentation m/z 209, 215 (standard), m/z 267, 270 (standard) and m/z 291, 296 (standard) were used for the quantification of JA, 12-OH-JA and OPDA, respectively.

Isolation of total RNA and cDNA synthesis

Total RNA was extracted from 150 mg of powdered leaf tissue using the Trizol method (Invitrogen, Carlsbad, CA, USA). Residual DNA was removed using 2U of DNase I (Fermentas GMBH, St. Leon-Rot, Germany) in a reaction for 30 min at 37°C. The RNA concentration was determined using a NanoDrop® ND-1000 spectrophotometer. Single-stranded cDNA was containing a piece of filter paper on which 100 μl of a 10% solution of JAME in ethanol was spotted (Chen et al. 2002). JAME treatment was repeated every 24 h for three or four consecutive days.

Insect bioassay

Cotton leafworms (S. littoralis Boisduval) were selected from a continuous laboratory culture (Laboratory of Agrozoology, Ghent University, Belgium). Larvae were reared on an artificial diet under standard conditions of 25°C, 65% relative humidity and a 16 h photoperiod as described (Smagghe and Degeest 1994, Smagghe et al. 2005). One insect of the fourth instar was placed on one leaf of a 16-week-old greenhouse-grown tobacco plant. Each assay was performed in triplicate. After feeding for 12 h, the insects were removed. Wounded and systemic (untreated) leaves were tested immediately after insect removal for agglutination. The leaf material was powder in liquid nitrogen using a pestle and mortar and stored at −80°C for later use. Different samples were analyzed for Nictaba expression at the RNA level using RT-PCR as well as at the protein level using agglutination assays and Western blots.

Alternatively, one leaf of a tobacco plant was subjected to insect herbivory for different time periods (3–24 h). The leaf material was collected 48 h after the start of the experiment and analyzed for lectin activity as described above.
synthesized from 1 μg of total RNA using MMLV reverse transcriptase (Invitrogen).

**RT–PCR**

RT–PCR was performed on single-stranded cDNA using a nested PCR with different sets of primers according to Sambrook et al. (1989). Amplification of the Nicataba sequence (Genbank accession No. AF389848) was achieved using primers evd 42 and evd 43 in a first reaction, and L35 and L36 in a second reaction, as described by Lannoo et al. (2006a). To amplify the tobacco AOC sequence (Genbank accession No. AJ308487), a nested PCR was performed using primers evd 231 (5’TATGCCACGTCTCCCTCA GGC3’) and evd 232 (5’TCAATTAGTAAATTCCTCAGT GC3’) in a first reaction followed by a second PCR using primers evd 246 (5’CCCAATCTCTTTAACTCGGC3’) and evd247 (5’CAAGATAGTGTCCTGTAAATGC3’). As a control for the RT–PCR, the ribosomal protein L25 (Genbank accession No. L18908) was used and amplified using primers evd 282 (5’TGAATGAAGATGGAGAAGACAAC3’) and evd 283 (5’CCATCAAATGTATCTCTAATAATGTCACAG3’) as described by Volkov et al. (2003). RT–PCR was performed in an AmplifierTM Thermolyne apparatus (Dubuque, IA, USA) using Taq polymerase (Invitrogen) according to the manufacturer’s instructions. For all RT–PCRs, the following program was used: 2 min at 94°C followed by 12–15 cycles of 15 s at 94°C, 30 s at 50°C and 60 s at 72°C, and a final incubation for 5 min at 72°C.

**Preparation of crude extracts**

Leaves were homogenized in 20 mM 1,3-propane diamine (5 ml buffer per gram FW leaf material) with a mortar and pestle. The homogenates were transferred to centrifuge tubes and centrifuged at 3,000 rpm for 10 min at 4°C until use.

**Agglutination assay**

To check the lectin activity in crude extracts, agglutination assays with trypsin-treated rabbit erythrocytes were performed in glass tubes by mixing 10 μl of crude extract with 10 μl of 1 M ammonium sulfate and 30 μl of a 2% solution of rabbit erythrocytes (made up in phosphate-buffered saline containing 137 mM NaCl, 8 mM Na2HPO4, 2 mM KH2PO4). Agglutination was assessed visually after 10 min at room temperature. To estimate the lectin content semi-quantitatively, extracts were serially diluted in 1 M ammonium sulfate with 2-fold increments. Aliquots of 10 μl of the diluted extracts were transferred into polystyrene 96 U-welled microtiter plates and centrifuged at 1,200 × g for 10 min. The homogenates were visualized by staining with Coomassie brilliant blue or peroxidase-coupled goat anti-rabbit IgG (Dako A/S, Denmark) as the secondary antibody. Immunodetection was achieved by a colorimetric assay essentially as described by Wang et al. (2003).

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**References**


Jasmonate-induced expression of tobacco lectin


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