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Spodoptera Littoralis Induced Lectin Expression in Tobacco

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Abstract

The induced defense response in plants towards herbivores is mainly regulated by jasmonates and leads to the accumulation of so-called jasmonate-induced proteins. Recently, a jasmonate (JA) inducible lectin called *Nicotiana tabacum* agglutinin or NICTABA was discovered in tobacco (*N. tabacum* cv Samsun) leaves. Tobacco plants also accumulate the lectin after insect attack by caterpillars. To study the functional role of NICTABA, the accumulation of the JA precursor OPDA, JA as well as different JA metabolites were analyzed in tobacco leaves after herbivory by larvae of the cotton leafworm (*Spodoptera littoralis*) and correlated with NICTABA accumulation. It was shown that OPDA, JA as well as its methyl ester can trigger NICTABA accumulation. However, hydroxylation of JA and its subsequent sulfation and glucosylation results in inactive compounds that have lost the capacity to induce NICTABA gene expression. The expression profile of the *NICTABA* after caterpillar feeding was recorded in local as well as in systemic leaves, and compared to the expression of several genes encoding defense proteins, and genes encoding a tobacco systemin and the allene oxide cyclase, an enzyme in JA biosynthesis. Furthermore, the accumulation of NICTABA was quantified after *S. littoralis* herbivory and immunofluorescence microscopy was used to study the localization of NICTABA in the tobacco leaf.

Keywords: agglutinin, carbohydrate binding, lectin, plant insect interaction, *Nicotiana tabacum*, Nictaba

Introduction

Plants are immobile and therefore unable to avoid hostile visits by pest insects or pathogens. To protect themselves against these threats, plants have developed a wide range of mechanical and chemical defense mechanisms to prevent or counteract injury by potential attackers. Although defense mechanisms are beneficial for plants in the presence of herbivores, plant resistance can be costly in the absence of enemies. When part of the limited resources is constantly invested in defense, the plant is restricted in its growth and reproduction (Zavala and Baldwin 2004). Consequently, inducible defense mechanisms have evolved which allow plants to use resources for defense purposes only when they are under attack. The induced defense response in plants towards herbivores is characterized by an alteration of the gene expression profile, which leads to accumulation of newly synthesized proteins.

Jasmonates are important signal molecules of plant responses to abiotic and biotic stresses as well as in plant development. They regulate induced defense mechanisms in plants after insect attack and wound response in general (Wasternack et al. 2006). Jasmonates comprise jasmonic acid (JA), its methyl ester (Me-JA), conjugates with amino acids, hydroxylated JA such as 11-OH-JA and 12-OH-JA as well as the glucoside and sulfate of 12-OH-JA (12-*O*-Glc-JA, 12-HSO_4-JA) (Wasternack 2007). Furthermore, the precursor of JA, 12-oxophytodienoic acid (OPDA) is of importance exhibiting JA-independent signaling properties at least in barley (*Hordeum vulgare*) leaves and Arabidopsis (Kramell et al. 2000; Stinzi et al. 2001; Mueller et al. 2008; Ribot et al., 2008). A characteristic feature of the wound response is an endogenous rise in OPDA, JA and its metabolites (Schilmiller and Howe 2005; Wasternack et al. 2006; Miersch et

al. 2008), followed by local and systemic expression of JA-induced genes. Both JA and Me-JA are well known inducers of local and systemic defense responses in many species. There was some debate whether Me-JA acts as a real endogenous signaling molecule, but transgenic approaches revealed that Me-JA is active only after hydrolysis (Kessler et al. 2004; Taki et al. 2005; Wu et al. 2008). Most recently, (+)-*7-iso*-jasmonoyl-L-isoleucine was identified as the endogenous bioactive jasmonate, and epimerization and methyl esterification were shown to inactivate its biological activity (Fonseca et al., 2009). In addition, hydroxylation and sulfation of JA leads to a partial switch off in JA signaling in tomato (*Solanum lycopersicum*) (Miersch et al. 2008).

Most of the newly synthesized jasmonate-induced proteins (JIPs) fall into two functional groups that include (i) signal pathway enzymes, and (ii) antinutritional proteins such as proteinase inhibitors (PIs), polyphenol oxidase (PPO) or lectins. The first group, including products of genes that are expressed immediately after herbivore attack, has no direct deleterious effect on its enemies, but regulates the speed and intensity of the defense response towards its attackers. They are mostly enzymes involved in the oxylipin pathway (i.e. synthesis pathway for jasmonates) such as lipoxygenase (LOX), allene oxide synthase (AOS) or allene oxide cyclase (AOC) (Wasternack 2007), proteins involved in JA signaling such as JAZ proteins (Chini et al. 2007; Thines et al. 2007; Chung et al. 2008), or transcription factors that regulate JIP gene expression like MYC2 (Lorenzo et al. 2004; Dombrecht et al. 2007). In contrast to these proteins many newly synthesized proteins of the second group have a direct defense function towards the attackers. Among them, the best-studied examples are the PIs which target the major proteolytically active digestive enzymes in the midgut of herbivores. In this way PIs slow down the growth and increase the mortality of lepidopteran herbivores in the larval stage (Glawe et al. 2003; Zavala et al. 2004). Another class of direct defense proteins consists of plant lectins, found

in many plant species including many agricultural crops (Van Damme et al. 1998; 2007). Plant lectins comprise all proteins possessing at least one non-catalytic domain that binds reversibly to specific mono- or oligosaccharides (Van Damme et al. 1998). Since the specificity of these binding motifs is primarily directed against foreign glycans, it is assumed that many lectins are involved in the recognition and binding of glycans from foreign organisms, and thus play a role in plant defense. At present especially lectins that occur at high amount in tissues in which they represent up to 10 % of the total protein content have been studied. Because of their preferential accumulation in vegetative storage tissues and seeds it is believed that these plant lectins function as storage proteins that can act as defense proteins when the plant is under attack (Van Damme et al. 1998; 2007).

Recently it has been shown that some plant tissues, which apparently lack lectin activity under normal physiological conditions, start to accumulate plant lectins under stress conditions like drought, high salt, wounding, treatment with plant hormones or insect attack (Zhang et al. 2000; Chen et al. 2002; Van Damme et al. 2008). Although only limited inducible plant lectins have been characterized in some detail, sequence analyses indicate that this new group of lectins is very abundant in the plant kingdom (Van Damme et al. 2008). These lectins distinguish themselves from the classical lectins by their cellular localization and their relatively low concentrations. One of these inducible plant lectins has been identified in leaves of *Nicotiana tabacum* (var Samsun NN) (Chen et al. 2002). This lectin called *Nicotiana tabacum* agglutinin or NICTABA is synthesized after treatment with certain jasmonates, whereas no NICTABA accumulation can be found after treatment with other plant hormones making this tobacco lectin a genuine JIP. NICTABA has been purified and characterized as a dimeric protein composed of two unglycosylated subunits of 19 kDa. Originally, a specificity towards oligomers of N-acetylglucosamine was addressed to NICTABA, but a more detailed inspection using glycan

arrays revealed that NICTABA preferentially recognizes high mannose and complex N-glycans (Chen et al. 2002; Lannoo et al. 2006b).

Recently, it was shown that NICTABA is also induced in tobacco leaves by insect herbivory (Lannoo et al. 2007). It is known, however, that one of the plant responses to chewing insects includes an increase of the endogenous amount of JA and related compounds, and the accumulation of different defense proteins (Howe and Jander 2008; Wang et al. 2008). In this paper a temporal and spatial analysis was set up to study the levels of different jasmonates after insect herbivory and the concurrent expression of *NICTABA* and other genes encoding JIPs. The accumulation of the JA-precursor OPDA, JA as well as hydroxylated, sulfated and glucosylated JA metabolites was recorded in local and systemic leaves after herbivore attack by the cotton leafworm *Spodoptera littoralis* (Boisduval) (Lepidoptera, Noctuidae), a polyphagous pest insect important in agriculture. Clear differences were observed in the temporal and spatial dynamics for the different jasmonates related to NICTABA accumulation during *S. littoralis* herbivory. Among the various jasmonates OPDA seems to be preferentially active in NICTABA accumulation, whereas 12-OH-JA, 12-HSO_4-JA and 12-*O*-Glc-JA were inactive. Our results show the importance of JA-metabolism in the formation of the tobacco protein NICTABA.

Results

Kinetics of accumulation of different jasmonates in tobacco after herbivory

Caterpillars were allowed to feed continuously from a tobacco leaf for up to 16 h. At different time points the amounts of OPDA, JA, 11-OH-JA, 12-OH-JA, 12-HSO_4-JA and 12-*O*-Glc-JA were quantified in the attacked leaves and compared to control leaves (Fig. 1). For the control leaves no changes were detected in jasmonate levels. In contrast, clear changes were recorded for jasmonate levels in tobacco leaves subjected to caterpillar attack. An accumulation of free OPDA started within the first 30 minutes of herbivory. OPDA levels increased continuously starting from 90 ± 33 pmol g^{-1} FW until maximum levels of 1,582 ± 101 pmol g^{-1} FW were reached at the end of the feeding period (Fig. 1A). Consequently, herbivory resulted in a 17-fold increase after 16 h of continuous herbivory compared to starting levels. JA levels were close to the detection limit before herbivory (24 ± 25 pmol g^{-1} FW), but also increased conspicuously within the first 30 minutes of caterpillar feeding (Fig. 1B). Unlike OPDA, the JA level reached its maximum after 3 h of continuous herbivory (1,954 ± 330 pmol g^{-1} FW). This is a more than 80-fold increase compared to JA levels present before herbivory. After this initial increase, the JA levels decreased again but remained 25 to 50 fold higher than in control leaves. Once the JA level passed its maximum, a continuous accumulation of 12-OH-JA appeared reaching a level of 8,708 ± 1,137 pmol g^{-1} FW after 16 h of herbivory while 12-OH-JA levels before herbivory were 121 ± 46 pmol g^{-1} FW (Fig. 1C). This represents a 72-fold increase and a 7-fold higher level than JA level at that time. Levels of 11-OH-JA were 868 ± 91 pmol g^{-1} FW after 16 h compared to 45 ± 23 pmol g^{-1} FW before herbivory representing a 19-fold increase but about 10-fold lower than

levels for 12-OH-JA (Fig. 1D). After 8-12 h of continuous caterpillar feeding, increasing levels of the metabolites 12-HSO_4-JA (5,444 ± 2,123 pmol g^{-1} FW after 16 h) and 12-*O*-Glc-JA (21,147 ± 1,446 pmol g^{-1} FW after 16 h) were also measured, resulting in an 142- and 520-fold increase, respectively, compared to starting levels (Fig. 1E and 1F). 12-*O*-Glc-JA was the most abundant jasmonate compound after 16 h herbivory exceeding the JA level by more than one order of magnitude.

Jasmonate levels were analyzed not only in the leaf under attack, but also in the leaf phyllotactically three positions above the attacked leaf. This leaf was chosen to assure a good vascular connectivity between the systemic leaf and the attacked leaf. In contrast to the leaf with herbivory, no significant changes were detected for jasmonates in the systemic leaves ($p < 0.05$).

Comparing all accumulation kinetics three characteristic features are obvious: (i) During herbivory a transient accumulation of JA, but a steady increase in OPDA, 11-OH-JA, 12-OH-JA, 12-HSO_4-JA and 12-*O*-Glc-JA was recorded in the tobacco leaf (Fig. 2). (ii) The accumulation of the different jasmonates in the tobacco leaf during herbivory is time-dependent. OPDA as well as JA start to accumulate already in the first 30 minutes, while accumulation of the hydroxylated, glucosylated and sulfated jasmonates start only after 3, 8 and 12 hours, respectively (Fig. 2). (iii) No significant changes in levels of jasmonates were measured in systemic leaves.

Activity of different jasmonates in NICTABA accumulation

To analyze the putative link between an endogenous rise in jasmonate levels after insect herbivory and NICTABA accumulation, the kinetics of NICTABA accumulation was recorded using an ELISA assay (Fig. 3). Treatment of tobacco leaves with OPDA and JA resulted in a maximal NICTABA accumulation of 1,155 ± 301 ng g^{-1} FW and 1,346 ± 117 ng g^{-1} FW,

respectively, three days after application (Fig. 3). When tobacco leaves were treated with Me-JA, maximal NICTABA accumulation was observed one day earlier (1,630 ± 32 ng g^{-1} FW) (Fig. 3). After 3-4 days no significant differences could be detected between the maximal NICTABA levels after OPDA, JA and Me-JA treatment (Fig. 3). Interestingly, treatments with 12-OH-JA, 12-HSO_4-JA and 12-*O*-Glc-JA did not result in NICTABA accumulation.

In addition, the lectin activity in the leaf extracts was measured using an agglutination assay. All protein extracts obtained after application of OPDA, JA or MeJA showed clear agglutination of red blood cells suggesting the presence of a lectin, whereas protein extracts from leaves treated with 12-OH-JA, 12-HSO_4-JA and 12-*O*-Glc-JA did not show any detectable agglutination activity. SDS-PAGE and Western blot analyses confirmed this observation (Supplemental Fig. S1). Three days after treatment with OPDA, JA and Me-JA, a clear protein band of 19 kDa is detected on the SDS-PAGE, indicating the presence of NICTABA, while treatment with 12-OH-JA, 12-HSO_4-JA and 12-*O*-Glc-JA did not show a comparable protein band (Supplemental Fig. S1A). Western blot analysis clearly confirmed the accumulation of NICTABA only after treatment with OPDA, JA and Me-JA (Supplemental Fig. S1B).

Expression of *NICTABA* after herbivory

RT-PCR analysis was performed to evaluate the temporal correlation between herbivory by *S. littoralis*, jasmonate levels and transcript accumulation of genes encoding NICTABA and several defense proteins. Transcripts of *NICTABA* in the attacked leaf are clearly detectable after 10 h of herbivory (Fig. 4). Similar kinetics was found for *TRYPSIN PROTEINASE INHIBITOR* (*NtTPI)*, whereas the *NtAOC* transcript levels were clearly enhanced after 4 h of herbivory. The mRNA for the *HYDROXYPROLINE-RICH GLYCOPEPTIDE SYSTEMIN PRECURSOR* (*ppTobHS-A)* is

already detectable without herbivory but is upregulated after herbivory. The transcript levels of the recorded genes were similar in the systemic leaf three positions above the attacked leaf, except for *NtAOC*, which was clearly expressed to a lower extent and at a later time point in the systemic leaf.

Quantification of NICTABA accumulation after herbivory in the attacked and the systemic leaf

One *S. littoralis* larva was allowed to feed on a tobacco plant for a period of 15 h. Afterwards caterpillars were removed and protein extracts were made at different time points during the next period of 36 h. As shown in Fig. 5, a NICTABA level of 2,079 ± 871 ng g^{-1} FW was measured after 15 h of herbivory and reached a maximum level of 5,473 ± 1,156 ng g^{-1} FW approximately 24 h after the caterpillar was removed. These results are confirmed by the Western blot analyses (Supplemental Fig. S2). Since *NICTABA* transcripts accumulated clearly in the systemic leaf (Fig. 4), the accumulation of NICTABA was also quantified in the leaves below and above the leaf attacked by *S. littoralis* (Fig. 5). NICTABA was detected in all analyzed leaves indicating a systemic signaling to younger as well as to older leaves. After the initial 15 h of herbivory, no significant increase for NICTABA was detectable in the systemic leaves. However starting from 12 h after removal of the larvae, NICTABA was clearly accumulating also in these leaves. Maximum levels for NICTABA were detected in the leaf three positions above the attacked leaf and reached 3,543 ± 892 ng g^{-1} FW approximately 24 h after ending of the herbivory period. However, at all time points, NICTABA levels were significantly lower in the systemic leaves compared to the leaf with herbivory.

The accumulation of NICTABA was compared to the increase of PI activity after insect herbivory. Using a radial diffusion assay the increase in TPI and CPI activity was recorded in leaves with herbivory (Supplemental Fig. S3). TPI and CPI activity in control tobacco leaves was 39 ± 1.5 µg g^{-1} FW and 4.4 ± 3.6 µg g^{-1} FW, respectively. After an initial feeding period of 15 h, *S. littoralis* caterpillars were removed and TPI and CPI activity was recorded in leaf extracts at different time points for the subsequent 36 h. Maximal levels for TPI and CPI reached 269 ± 16 µg g^{-1} FW and 40.7 ± 26 µg g^{-1} FW, respectively, after 36 h, resulting in a 7-fold and a 9-fold increase for TPI and CPI, respectively, compared to control levels.

NICTABA occurrence after caterpillar herbivory is restricted to the leaf parenchyma cells

To study the tissue specific distribution of NICTABA under non-induced and herbivore/Me-JA-induced conditions immunofluorescence microscopy was performed. Using a specific antibody directed against NICTABA the occurrence of the lectin in the attacked leaf as well as in a systemic leaf was clearly demonstrated (Fig. 6A). Whereas NICTABA was detected in most palisade and spongy parenchyma cells of the attacked leaf, the lectin was mainly detectable in the spongy parenchyma cells and lower epidermis cells in the systemic leaf. No NICTABA protein was detected in the vascular bundles. At the cellular level, the previously reported occurrence of NICTABA in the cytoplasm and the nucleus of leaf cells was confirmed (Chen et al., 2002). The distribution pattern observed for NICTABA contrasts to that of NtAOC in non-treated tobacco leaves. Using a specific antibody directed against SlAOC from tomato (Ziegler et al. 2000), the occurrence of NtAOC was visualized in the plastids of vascular tissues as well as in the epidermal cells of the tobacco leaf (Fig. 6B).

Discussion

The increase of jasmonate levels and the expression of wound-inducible genes after herbivory is a common phenomenon known for many plant systems (Qu et al. 2004; Schmidt et al. 2004). One of these herbivore and JA-induced genes is NICTABA which encodes a lectin. Interestingly, due to the absence of a functional lectin gene NICTABA is not expressed in *N. attenuata* (Lannoo et al. 2006a), where many data on herbivore-plant interactions and accumulation of jasmonates were collected (Halitschke and Baldwin 2004; Wang et al. 2008). Consequently, it can be concluded that herbivore-induced responses can be different even in two species of the same plant family. Here, we report on a temporal and spatial link between endogenous levels of different jasmonates, defense gene expression and NICTABA accumulation in *N. tabacum* during *S. littoralis* herbivory.

Our results show that herbivory by *S. littoralis* provokes a rapid increase of OPDA and JA levels. In contrast to the transient peak in JA level observed after single wounding events, no fast decline to the basal JA level is observed after insect attack (Lou and Baldwin 2003; Strassner et al. 2002; Wu et al. 2006). During further insect feeding a relatively high JA level is maintained in the attacked tobacco leaves (compared to control leaves), while the OPDA level kept increasing (Fig. 1). This can be explained if insect herbivory is considered to be a series of sequential mechanical wounds (Maffei et al. 2007). A similar scenario was also demonstrated in lima bean, where the systemic release of defensive volatiles could be induced by the use of a mechanical wounding apparatus that mimicked the feeding behavior of a caterpillar (Mithöfer et al. 2005).

Although JA and its methyl ester were previously shown to induce NICTABA, more detailed analyses reported in this paper support the idea that OPDA itself may fulfill this role as indicated

by its abundant accumulation upon herbivory (Fig. 1A) and its ability to upregulate the accumulation of NICTABA (Figs. 2 and 3). Exogenous application of several jasmonates revealed that only OPDA, JA and Me-JA are active for inducing NICTABA while hydroxylated or sequentially processed JA had no activity. Although the observed NICTABA accumulation after OPDA treatment might be caused by JA formed during this treatment, the amount of JA is usually not more than 50 % of OPDA (Delker et al. 2007). OPDA is among the early and steadily increasing oxylipins accumulating after continuous herbivory (Fig. 1). Interestingly, the abundant accumulation of OPDA occurs after the peak of JA accumulation and exceeds that of JA 1.4-fold at 16 h (Fig. 1A versus 1B) and was accompanied by *NICTABA* expression, which was only detectable after 10 h of herbivory (Fig. 2). Although JA and its JA-isoleucine conjugate are generally regarded as the most important signaling molecules in the octadecanoid pathway (Staswick 2008), several reports have addressed a differential inducing potential to OPDA and JA. Both compounds can act separately to coordinate the expression of different sets of genes (Stinzi et al. 2001; Weber et al. 2002; Taki et al. 2005; Mueller et al. 2008; Ribot et al. 2008). OPDA was also shown to induce the synthesis of certain diterpenoid volatiles in lima bean that could not be induced by JA, while soybean accumulated more phytoalexins after treatment with OPDA compared to JA (Koch et al. 1999; Fliegmann et al. 2003). Stinzi et al. (2001) demonstrated that mutant *Arabidopsis* plants, which fail to accumulate biologically active JA, maintain their ability to induce defense responses against insects and fungi. Moreover, the reduced hypocotyl cell expansion in the *det3* mutant is caused by an OPDA- but not a JA-mediated growth inhibition (Brüx et al. 2008). Finally, some genes of the *PHO* gene cluster are specifically expressed in response to OPDA (Ribot et al. 2008).

After exogenous jasmonate treatment of tobacco leaves, maximum NICTABA accumulation was reached one day earlier after treatment with Me-JA compared to the treatment with OPDA or

JA suggesting that Me-JA is the most active jasmonate. However, recent results using wild tobacco (*Nicotiana attenuata*) indicate that Me-JA is demethylated into JA before triggering defense responses (Wu et al., 2008). In addition, the permeability of Me-JA may enhance distribution in the leaf resulting in a faster response.

After 4 h of caterpillar feeding, JA levels remained more or less at the same level, while 11-OH-JA and 12-OH-JA levels increased steadily (Fig. 1). Similar kinetics of wound-induced accumulation of various hydroxylated jasmonates was found recently in *A. thaliana* leaves (Glauser et al. 2008). In wounded tomato leaves a JA-dependent accumulation of 12-OH-JA is followed by the accumulation of 12-HSO_4-JA and 12-*O*-Glc-JA (Miersch et al. 2008). A similar kinetics appeared upon continuous herbivory of tobacco leaves (Fig. 1). This raises the question on biological activity of these JA metabolites. In *N. tabacum*, 12-OH-JA, 12-HSO_4-JA or 12-*O*-Glc-JA do not induce NICTABA accumulation when applied exogenously (Fig. 3). The inability of 12-OH-JA and its sulfated and glucosylated derivatives to induce NICTABA accumulation suggests an inactivation mechanism, in which the biologically active compound JA can be converted to 12-OH-JA and further metabolites, which, in turn, can change certain physiological responses in tobacco such as e.g. the down regulation of *NICTABA* expression. A curious scenario seems to exist in JA biosynthesis and signaling: On the one hand, there is the well-established positive feedback in JA biosynthesis by JA (for review see Wasternack 2007). On the other hand, JA biosynthesis is followed by a JA-dependent formation of 12-OH-JA, which is inactive at least in terms of activation of subsets of genes (Miersch et al. 2008; and data shown here). Consequently, there seems to be a time-dependent shift in generation of active and inactive jasmonates. A similar regulatory loop was most recently described by the JA-inducible expression of JAZ proteins which act as negative regulators of JA-induced gene expression (cf. reviews of Katsir et al. 2008; Staswick 2008).

In order to analyze the JA-dependence of NICTABA formation, its accumulation was compared with the expression of genes coding for several well-characterized JIPs like NtTPI, NtAOC and ppTobHSA. While the NtAOC is an important enzyme in jasmonate biosynthesis, ppTobHSA represents the tomato systemin homologue in tobacco (Pearce et al. 2001), being also induced by chewing insects (Rocha-Granados et al. 2005) and thought to be involved in defense gene expression (Schilmiller and Howe 2005). Because a clear upregulation of NtAOC could only be detected after 2 h of herbivory (Fig. 4), while increase in JA levels starts within the first hour (Fig. 1), another way for JA synthesis possibly exists that is not mediated by AOC gene expression. A similar temporal expression was also observed for the allene oxide synthase in *N. attenuata* (Ziegler et al., 2001). The expression of the genes encoding JA biosynthesis enzymes starts within the first hours after wounding or herbivory which led to the term 'early genes' (Ryan, 2000), whereas defense genes like PIs were designated as 'late genes'. The results presented here clearly show that *NICTABA* has an expression pattern similar to that of the late genes. As shown in Fig. 4, the expression of the *NtTPI* as well as *NICTABA* genes is clearly visible 10 h after the start of caterpillar attack. Although NICTABA accumulation can be detected after 15 h, it still takes another 24 h before its maximal protein levels appeared (Fig. 5). The time of expression as well as the high level favor a putative role of NICTABA in tobacco defense mechanisms rather than a function in the JA-dependent signaling pathway. Although NtTPI and NICTABA have a similar induction pattern, there are some differences in the regulation of their expression. Herbivore elicited expression of genes encoding PI or TPI is known to be regulated especially by JA, but also ethylene and abscisic acid (ABA) are involved (Koiwa et al. 1997). In contrast, NICTABA induction is regulated only by certain jasmonates, while other plant hormones cannot induce the tobacco leaf lectin (Lannoo et al. 2007).

A systemic induction of NICTABA is shown in the tobacco leaves, but no corresponding increase in jasmonate levels could be recorded in the systemic leaves. The latter agrees with earlier reports on JA and OPDA levels in systemic leaves of wounded tomato plants (Strassner et al. 2002). Furthermore, grafting experiments conducted with several mutant tomato plants showed that jasmonate perception but not jasmonate biosynthesis is necessary for a systemic expression of PI genes, whereas the generation of a systemic signal needs local jasmonate biosynthesis (Stratmann 2003; Schilmiller and Howe 2005; Wasternack et al. 2006). These experiments strongly suggest that JA or a JA related compound act as a long distance signal in the systemic defense response. Long distance transport has been shown with labeled jasmonates (Thorpe et al. 2007). The observation that NICTABA is expressed in distal leaves without significant increase in jasmonate levels, suggests that there are unknown signals downstream of jasmonate biosynthesis necessary for NICTABA accumulation. Thus NICTABA represents another example of a gene that is systemically expressed after local generation of jasmonates following herbivory.

The accumulation of AOC is confined to the vascular bundles in tobacco (Fig. 6B) and tomato (Hause et al. 2000; Stenzel et al. 2003) and occurs in sieve elements (Hause et al. 2003), while the gene encoding *PROSYSTEMIN* is exclusively expressed in the vascular phloem parenchyma cells of tomato leaves (Narváez-Vásquez and Ryan 2004). In contrast, the most prominent herbivore response gene product PI accumulates exclusively in mesophyll cells. Since the location of herbivore-induced gene products can be diverse we also investigated the tissue distribution of NICTABA. Immunocytochemical analyses clearly showed that the accumulation of NICTABA after insect herbivory is restricted to the cytoplasm and the nucleus of the palisade and the spongy mesophyll cells of tobacco leaves. This corresponds with the location of herbivore-induced PIs in mesophyll cells of tomato leaves (Schilmiller and Howe 2005). Both PI

and NICTABA were never detected in vascular bundles. Obviously, NICTABA represents a typical late defense gene also with respect to its location in the mesophyll cells apart from the vascular bundles. This might be important in view of a possible role of NICTABA in plant defense against herbivores which feed first in leaf areas away from vascular bundles.

Materials and Methods

Plant material

Tobacco (*N. tabacum* L. cv Samsun NN) seeds were germinated in Petri dishes filled with pot soil. After appearance of the cotyledons and first leaves plantlets were transferred to pots (10 cm diameter) filled with soil and kept in a growth chamber at 22°C, 70% relative humidity and a 16 h photoperiod.

Insects

The larvae from the cotton leafworm (*S. littoralis*) were selected from a continuous laboratory culture. Larvae were reared on an artificial diet under standard conditions of 25°C, 65% relative humidity and 16 h photoperiod as described by Hakim et al. (2007).

Treatment of tobacco leaves with jasmonates

Tobacco leaves from 8-week old tobacco plants were treated with OPDA, JA, Me-JA, 12-OH-JA, 12-HSO_4-JA or 12-*O*-Gluc-JA as described by Baldwin et al. (1996). For each compound 75 µg was dissolved in 10 µl lanolin paste resulting in final concentrations of 26 mM OPDA, 36 mM JA, 33 mM Me-JA, 33 mM 12-OH-JA, 25 mM 12-HSO_4-JA and 19 mM 12-*O*-Gluc-JA, respectively. Source and synthesis of jasmonates are described by Miersch et al. (2008). Control leaves were treated with 10 µl of pure lanolin. Tobacco leaves from three biological repeats were analyzed for 4 consecutive days.

Treatment of tobacco with *S. littoralis*

All the insect bioassays were performed with freshly molted (0-4 h after molting) *S. littoralis* caterpillars. Larvae of *S. littoralis* were placed onto a fully developed leaf of an 8-week old tobacco plant (unless stated otherwise) and caged with a plexiglass container to prevent the caterpillar from going to other leaves (Van de Veire et al. 1997). Each cage contained six air holes covered by net cloth for ventilation and one channel for the leaf petiole to allow enclosing a tobacco leaf without damaging the leaf. *S. littoralis* larvae were allowed to feed from the leaf for different time periods.

Quantification of endogenous jasmonate concentrations

Endogenous jasmonate concentrations in the tobacco leaves were determined and compared to control plants. For each feeding period six biological repeats were performed and leaves were

pooled into three groups. From each tobacco plant, the leaf with herbivory as well as the leaf at position three above the attacked leaf was collected immediately after caterpillar removal, powdered in liquid nitrogen using pestle and mortar, and stored at -80°C.

Extracts were made by homogenizing 500 mg of powdered plant material in 10 ml methanol after 100 ng ($^2H_6$) JA , 100 ng ($^2H_5$) OPDA, 100 ng 11-($^2H_3$)OAc-JA, 100 ng 12-($^2H_3$)OAc-JA, 250 ng 12-HSO_4-JA-($^2H_3$)Me ester and 12*O*-Glc-($^2H_7$)JA were added as internal standards. The homogenate was filtered and the eluent was evaporated followed by an acetylation step with 200 µl pyridine and 100 µl acetic acid anhydride at 20°C overnight. Then the extract was evaporated using absolute ethanol and dissolved in 3 ml ethyl acetate. The solvent was loaded on a Chromabond-SiOH-column (500 mg, Macherey-Nagel, Düren, Germany) and washed with an extra 3 ml ethyl acetate. The unbound fraction was evaporated and annotated as sample A. After elution of the column with methanol, the eluate was evaporated and annotated as sample B.

Sample A was dissolved in 10 ml of methanol and loaded on a DEAE-Sephadex A25 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) column (Ac^--form, methanol). After washing with 3 ml methanol, the column was eluted with subsequent 3 ml 0.1 M acetic acid in methanol, 3 ml 1 M acetic acid in methanol and 3 ml 1.5 M acetic acid in methanol. After evaporation, the eluate was dissolved in 110 µl methanol/water (1:1) and separated on a preparative HPLC column (Eurospher 100-C18, 5 µm, 250 x 4 mm) (Knauer, Berlin, Germany) using a gradient of 40% to 100% methanol in 25 min. Fractions at R_t 9.30 to 11.45 min (11- and 12-OH-JA), R_t 12.45 to 14.15 min (JA), R_t 18.00 to 19.15 min (dinor OPDA), and R_t 21.15 to 22.30 min (OPDA) were collected and combined. After evaporation the samples were dissolved in 200 µl chloroform/N,N-diisopropylethylamine (1:1) and derivatized with 10 µl pentafluorobenzylbromide at 20°C overnight. After evaporation the samples were dissolved in 5 ml n-hexane and loaded on a Chromabond-SiOH column (500 mg, Macherey Nagel). The

column was eluted with 7 ml n-hexane/diethylether (1:1, v/v), the samples were evaporated, dissolved in 100 µl acetonitrile and analyzed by GC-MS according to Miersch et al. (2008).

Sample B was dissolved in 100 µl methanol and 500 µl ethereal diazomethane was added for methylation. After incubation for 15 min at room temperature, the samples were evaporated, dissolved in 220 µl methanol/water (1:1, v/v), and the volume of the samples was reduced by vacuum centrifugation for 7 min. Then the samples were loaded on a preparative HPLC column (Eurospher 100-C18, 5 µm, 250 x 4 mm) (Knauer, Berlin, Germany) using a gradient of 10 % to 100 % methanol in 23 min. Fractions at R_t 10.25 to 11.55 min (12-HSO_4-JA-Me) and R_t 17.05 to 19.05 min (12-*O*-tetraacetyl-Glc-JA-Me) were collected and combined. After evaporation the samples were dissolved in 100 µl methanol and analyzed by LC-MS-MS according to Miersch et al. (2008).

Isolation of RNA and cDNA synthesis

One L4 *S. littoralis* larva was released onto a caged tobacco leaf and allowed to feed for different time periods up to 12 h. For each feeding period three independent analyses were performed on a leaf with herbivory as well as on the tobacco leaf at position three above the attacked leaf.

Total RNA was extracted from 200 mg of liquid nitrogen powdered leaf material using the Trizol method (Invitrogen, Carlsbad, CA) as described by the manufacturer. Residual DNA was removed by treatment with 2U of DNase I (Fermentas, St. Leon-Rot, Germany) in a 30 min reaction at 37°C. To check the RNA quality, equal amounts of RNA were loaded onto a 1.5 % agarose gel, separated by gel electrophoresis and visualized after ethidium bromide staining. The RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop

technologies, Wilmington, DE). Single stranded cDNA was synthesized starting from 1 μg of total RNA using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer instructions.

RT-PCR analysis

RT-PCR was performed on single stranded cDNA using Taq polymerase (Invitrogen, Carlsbad, CA) according to Sambrook et al. (1989). To amplify the *NICTABA* sequence (Genbank accession No AF389848) primers evd42 and evd43 as described by Lannoo et al. (2006a) were used. The *NICOTIANA TRYPSIN PROTEINASE INHIBITOR* (*NtTPI*) sequence (Genbank accession No DQ158189) was amplified using primers evd333 (5´-TTGGAATGTCTATGCTTGT-3´) and evd334 (5´-CAACCCTAGACTTCTGGAGATCA-3´), based on results from Rocha-Granados et al. (2005). The amplification of the tobacco *NtAOC* sequence (Genbank accession No AJ308487) was done using primers evd231 and evd232 as described in Lannoo et al. (2007), while the tobacco *HYDROXYPROLINE-RICH GLYCOPEPTIDE SYSTEMIN PRECURSOR A* (*ppTobHS-A*) sequence (Genbank accession No AY033148) was amplified using the primers as described by Rocha-Granados et al. (2005). The *RIBOSOMAL PROTEIN L25* (Genbank accession No L18908) was used as an internal control for the RT-PCR, and amplified using primers evd282 (5´-TGCAATGAAGAAGATTGAGGACAACA-3´) and evd283 (5´-CCATTCAAGTGTATCTAGTAACTCAAATCCAAG-3´) as described by Volkov et al. (2003). All RT-PCRs were performed in an AmplitronII Thermolyne apparatus (Dubuque, IA) using the following program: 2 min at 94°C followed by a variable number of 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. For *NICTABA*, *NtTPI*,

NtAOC, *ppTobHS-A* and the internal control, 25, 30, 40, 40 and 25 amplification cycles, respectively, were performed. For amplification of *ppTobHS-A* an annealing temperature of 47°C was used instead of 50°C for the other sequences.

Determination of agglutination and proteinase inhibitor activity

Agglutination and PI activity were measured in protein extracts made from tobacco leaves after herbivory by one L4 *S. littoralis* larva. After an initial herbivory period of 15 h, caterpillars were removed and leaf material was collected at different time periods up to 36 h after removal of the larvae (15 h until 51 h after the start of the experiment). A period of 15 h of herbivory was chosen based on the observation that after 12 h *NICTABA* expression could already be detected at the RNA level, whereas agglutination activity was not yet detectable (Lannoo et al. 2007). Leaves that were located above and below the corresponding attacked leaf were also harvested. For each time period three biological repeats were performed.

Extracts were made by homogenizing one gram tobacco leaves in 2.5 ml 20 mM 1,3-diaminopropane using mortar and pestle. The homogenates were centrifuged at 13,000 x g for 5 min and the supernatants were used as total protein extracts. Total protein content was measured using the Bradford method (Coomassie Protein Assay Kit, Thermo scientific, Rockford, IL) with NICTABA as protein standard. For each replicate, the average value of four subsamples was used to determine the soluble protein content.

The agglutination activity in protein extracts was analyzed using rabbit red blood cells (BioMérieux, Craponne, France) as described by Lannoo et al. (2007). The TRYPSIN PROTEINASE INHIBITION (TPI) and CHYMOTRYPSIN PROTEINASE INHIBITION (CPI)

activities were quantified using the radial diffusion assay protocol described by Van Dam et al. (2001).

NICTABA purification

NICTABA was purified from leaves of four to five month-old *Nicotiana tabacum* cv Samsun NN plants that were floating on a 50 µM Me-JA solution for 4 days. After jasmonate treatment a crude extract was made by mixing the tobacco leaves in 20 mM diaminopropane containing 10 mM thiourea to prevent oxidation. After centrifugation the extract was applied onto a Q Fast Flow column (HE Healthcare, Uppsala, Sweden) equilibrated with 20 mM diaminopropane. The column was washed with 20 mM diaminopropane and eluted using 0.1 M Tris-HCl buffer (pH 8.7) containing 0.5 M NaCl. The eluate was adjusted to pH 7 (with 1 M HCl) and applied onto a column filled with an acetylated chitin matrix. After washing of the chitin matrix with 0.2 M NaCl, the column was eluted using 20 mM diaminopropane. The eluate containing NICTABA was adjusted to 0.2 M NaCl and pH 7 (with 1 M HCl), and applied onto a column of ovomucoid-Sepharose 4B equilibrated with 0.2 M NaCl. NICTABA was desorbed using 20 mM diaminopropane. Finally, NICTABA was dialyzed and lyophilized, and stored for further use.

ELISA assay

To quantify the NICTABA content in protein extracts from tobacco leaves, microtiter plates (Maxisorp F96 Nunc, VWR, Leicestershire, UK) were coated overnight with 25 µg crude protein extracts or purified NICTABA serially diluted from 1 to 200 ng at 4 °C in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide; pH 9.6). The wells were

washed twice using phosphate buffered saline (PBS; 135 mM, NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.5) containing Tween20 (0.1 %, v/v) (PBST) and blocked with 5 % (w/v) non-fat milk powder (Applichem, Darmstadt, Germany) in PBS for 3 h at 37 °C. Afterwards, the wells were washed three times with PBST and incubated with affinity purified anti-NICTABA rabbit IgG (diluted 1:500 in PBS) for 1 h at 37 °C. After this incubation period, the wells were washed five times with PBST and incubated with horse radish peroxidase (HRP)-coupled goat anti-rabbit IgG (diluted 1:10,000 in PBS) (Sigma-Aldrich, Bornem, Belgium) for 1 h at 37 °C. Finally the wells were washed five times and the substrate buffer (0.4 mg/ml *O*-phenylenediamine hydrochloride (OPD) (Sigma-Aldrich, Bornem, Belgium) dissolved in 0.15 M citrate buffer (pH 5) with 0.04 % H_2O_2 (v/v)) was added. After incubation for 30 minutes in the dark, the absorbance was recorded at 450 nm using an automatic 96-well microtiter plate reader (Powerwave X340, Bio-Tek Instruments, VT).

Immunoblot analyses

Protein extracts from tobacco leaves were analyzed by SDS-PAGE in 15% acrylamide gels as described by Laemmli (1970). Proteins were visualized by staining with Coomassie brilliant blue or blotted onto polyvinylidene fluoride (0.45 µm) transfer membranes (Biotrace PVDF, PALL, Gelman Laboratory, Ann Arbor, MI). Immunoblot analysis was performed by blocking the membrane first with Tris-buffered saline (TBS; 10 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Triton X-100, pH 7.6) containing 5% (w/v) BSA, followed by consecutive incubation in TBS supplemented with a primary rabbit antibody directed against NICTABA (diluted 1:80), a HRP-coupled goat anti-rabbit IgG (diluted 1:300, Sigma-Aldrich, Bornem, Belgium) and the peroxidase-anti-peroxidase complex (diluted 1:400, Sigma-Aldrich). The immunodetection was

achieved using a 0.1 M Tris-HCl buffer (pH 7.6) containing 700 µM diaminobenzidine (DAB) and 0.03% (v/v) H_2O_2. The coloring reaction was stopped by washing with distilled water.

Immunolocalization studies of NICTABA and NtAOC in tobacco leaves

One L4 *S. littoralis* larva was released onto a caged leaf of a 7-week-old tobacco plant and allowed to feed for 12 h. Approximately 36 h after removal of the caterpillar small pieces of leaf material originating from areas at different distances from the leaf area that was damaged as well as from a systemic leaf three positions above the attacked leaf were collected. In addition, leaf pieces were also collected from tobacco leaves floated on a 50 µM Me-JA solution for 2 days and from non-treated leaves. The leaf material was fixed immediately with 4% (w/v) paraformaldehyde containing 0.1% (v/v) Triton X-100 in PBS using vacuum infiltration for 10 min and later for another 2 h at room temperature. After dehydratation in a graded series of ethanol, the specimen were infiltrated with polyethylene glycol (PEG) 1500 by incubation in ethanol solutions with increasing PEG concentration at 50°C until embedding in pure PEG according to Isayenkov et al. (2005). Cross sections (2 µm thick) were immunolabeled by incubation with affinity purified primary antibodies against NICTABA (diluted 1:150 in PBS containing 5% (w/v) BSA) or with polyclonal antibody against tomato AOC (Ziegler et al., 2000) (diluted 1:2000 in PBS containing 5% (w/v) BSA), followed by a goat anti-rabbit-IgG antibody conjugated with AlexaFluor488 (Molecular Probes, Leiden, The Netherlands). After immunolabeling, sections were stained with 0.1 µg/ml diamidino-2-phenylindole (DAPI) in PBS and analyzed with an epifluorescence microscope AxioImager (Zeiss, Jena, Germany) using the proper filter combinations. Micrographs were taken using AxioCam Mrc5 (Zeiss) and processed through Photoshop (Adobe).

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Legends to the figures

Figure 1. Kinetics of different jasmonates in tobacco leaves after caterpillar feeding for 16 h. Accumulation of OPDA (A), JA (B), 12-OH-JA (C), 11-OH-JA (D), 12-HSO_4-JA (E) and 12-*O*-Glc-JA (F) is shown in the leaf with herbivory (dark color) and in a systemic leaf three positions above the attacked leaf (light color). The data represent the mean (±SD) of three independent biological replicates. Different letters indicate statistically significant differences between jasmonate levels (ANOVA with Duncan's multiple range test; $p < 0.05$).

Figure 2. Schematic presentation of the accumulation of the different jasmonates and NICTABA in tobacco leaf tissue subjected to continuous herbivory. (A) Accumulation of jasmonic acid (JA) and 12-oxophytodienoic acid (OPDA). (B) Accumulation of 11-OH-JA, 12-OH-JA, 12-HSO4-JA and 12-*O*-Glc-JA.

Figure 3. Quantification of NICTABA accumulation in tobacco leaves. NICTABA levels were recorded for four consecutive days after treatment of the leaves with the JA-precursor OPDA, JA or the JA-metabolites Me-JA, 12-HO-JA, 12-HSO_4-JA and 12-*O*-Glc-JA. Each value represents the mean (±SD) of three independent biological replicates. Different letters indicate statistically significant differences among treatments (ANOVA with Duncan's multiple range test; $p < 0.05$).

Figure 4. Analysis of transcript accumulation of several jasmonate-responsive genes in tobacco leaves after herbivory by *S. littoralis* for 14 h. Transcript accumulation is shown in the attacked leaf (left panel) and the systemic leaf three positions above the leaf with herbivory (right panel).

Using RT-PCR, transcripts of the tobacco *HYDROXYPROLINE-RICH GLYCOPEPTIDE SYSTEMIN PRECURSOR A* (*ppTobHS-A*, 825 bp), the tobacco *ALLENE OXIDE CYCLASE* (*NtAOC*, 738 bp), *NICTABA* (608 bp) and the tobacco *TRYPSIN PROTEINASE INHIBITOR* (*NtTPI*, 1140 bp) were analyzed. The gene encoding the *RIBOSOMAL PROTEIN L25* (287 bp) was used as a control.

Figure 5. Local and systemic accumulation of NICTABA in tobacco leaves after *S. littoralis* herbivory. After an initial 15 h feeding period (time zero on the graph), *S. littoralis* larvae were removed and NICTABA levels were quantified for the next 36 h using an ELISA assay. Leaves positioned above or below the leaf under insect attack are indicated with + (1+ to 3+, to the top of the plant) and - (1- to 3- to the base of the plant), respectively. All data represent the mean (±SD) of three independent biological replicates. Different letters indicate statistically significant differences between NICTABA levels (ANOVA with Duncan's multiple range test; $p < 0.05$).

Figure 6. Immunolocalization of NICTABA in tobacco leaves after caterpillar attack and NtAOC in non-treated tobacco leaves. (A) Cross sections of 2 µm were made from a tobacco leaf (middle row) as well as from a systemic leaf (top row) 36 h after a 12 h period of herbivory. The leaf treated with Me-JA (bottom row) represents the positive control. All sections were immunolabelled with an antibody directed against NICTABA. (B) Cross sections of a non-treated tobacco leaf immunolabelled with an antibody against tomato AOC.

The different leaf sections were visualized for the immunodecorated protein (left column) and for DAPI-staining to visualize the nuclei of the leaf cells (middle column). The right column shows the overlay of the two previous images. Scale bars represent 20 µm (A) or 25 µm (B).

Supplemental data

Figure S1. Coomassie-stained SDS-PAGE and immunoblot of tobacco leaf extracts sampled 3 days after treatment with different jasmonates. (A) Coomassie stained SDS-PAGE illustrating NICTABA accumulation at 19 kDa after treatment with different jasmonates (indicated with an arrow). Lane 1 was loaded with a protein marker (PageRuler^{TM}, prestained protein ladder, Fermentas). Lanes 2 and 3 contain 25 µg of a tobacco protein extracted from leaves without treatment and 3 days after treatment with lanolin, respectively. Lanes 4-9 contain 25 µg of total leaf protein taken 3 days after treatment with OPDA, JA, Me-JA, 12-OH-JA, 12-HSO4-JA and 12-*O*-Glc-JA, respectively. Lane 10 was loaded with 2.5 µg of purified NICTABA. (B) Western blot analysis corresponding to the SDS-PAGE shown in (A). All lanes were loaded according to (A), except lane 10 was loaded with 250 ng of purified NICTABA.

Figure S2. Western blot for NICTABA accumulation in protein extracts from tobacco leaves during the first 36 h following a 15 h period of caterpillar herbivory. Lane 1 was loaded with 200 ng of purified NICTABA. Lanes 2 and 3 contain 25 µg of total protein from a tobacco leaf without herbivory (n.t. = non-treated) and a leaf collected after the initial herbivory period of 12 h, respectively. Lanes 4-8 contain 25 µg of total leaf protein taken 0 to 36 h after the initial herbivory period of 15 h.

Figure S3. Semi-quantitative analysis of PROTEINASE INHIBITORS (PI) after *S. littoralis* herbivory. Accumulation of TRYPSIN PROTEINASE INHIBITOR (TPI) and CHYMOTRYPSIN PROTEINASE INHIBITOR (CPI) was analyzed in protein extracts from tobacco leaves during the first 36 h following a 15 h period of caterpillar herbivory using a radial diffusion assay. The data represent the mean (±SD) of three independent measurements. Different letters indicate statistically significant differences between PI activity (ANOVA with Duncan's multiple range test; $p < 0.05$).

Figure 1

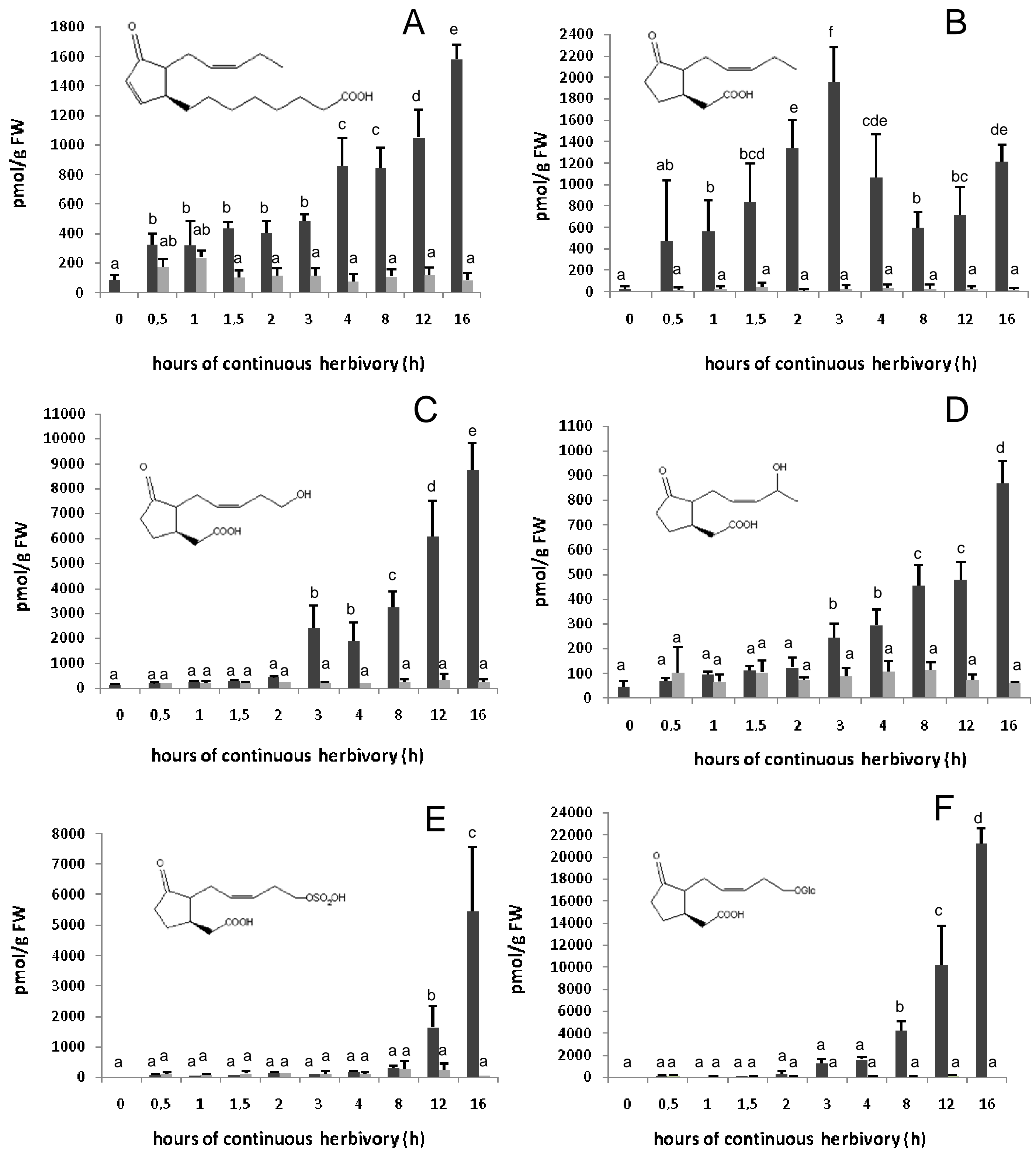

Figure 2

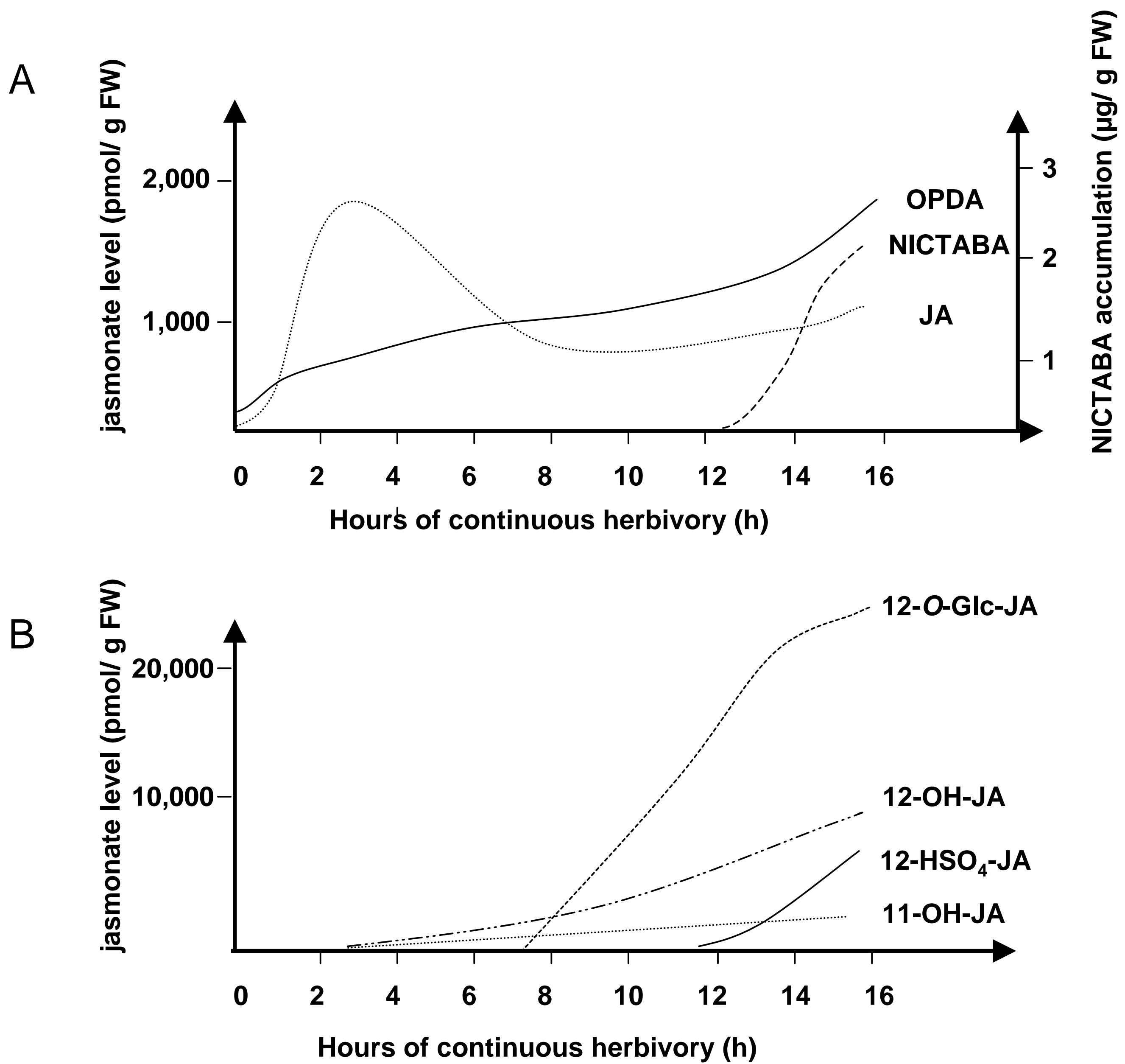

Figure 3

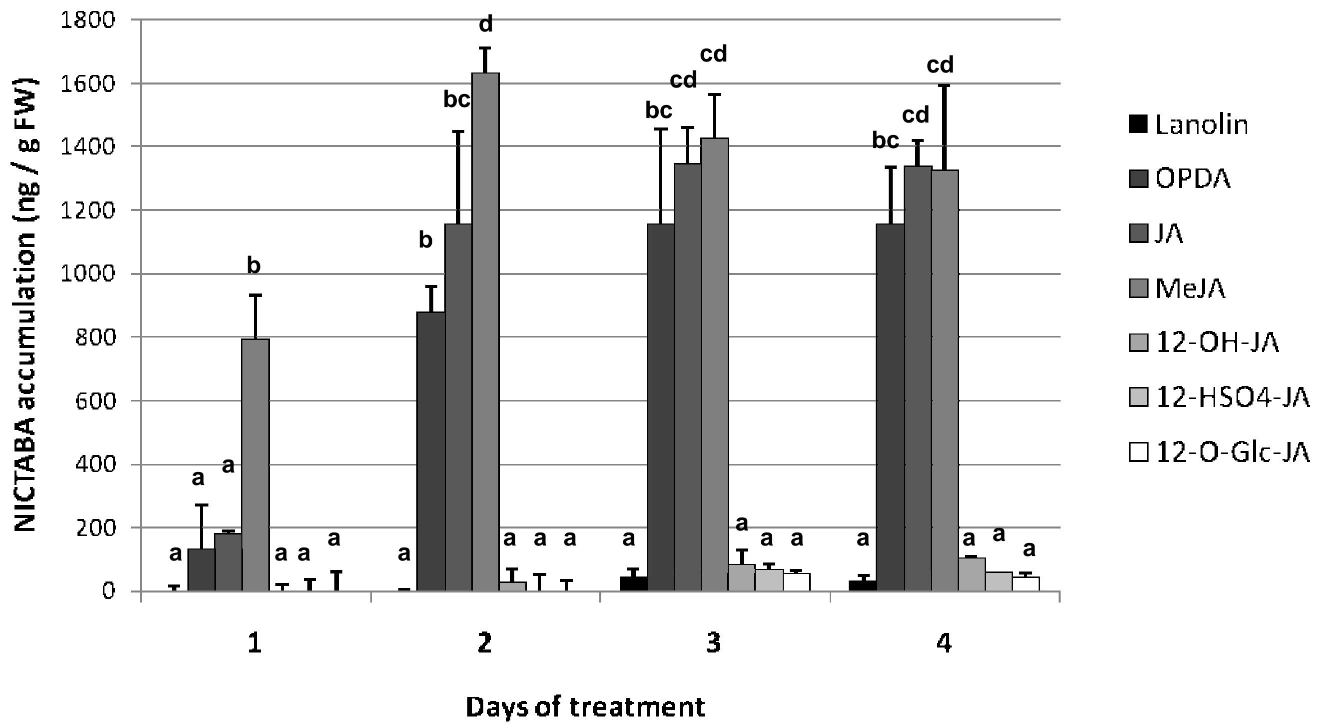

Figure 4

	0	2	4	6	8	10	12	14	0	2	4	6	8	10	12	14	(h)
ppTobHS-A																	40 cycles
Nt AOC																	40 cycles
NICTABA																	25 cycles
NtTPI																	30 cycles
control																	25 cycles

Figure 5

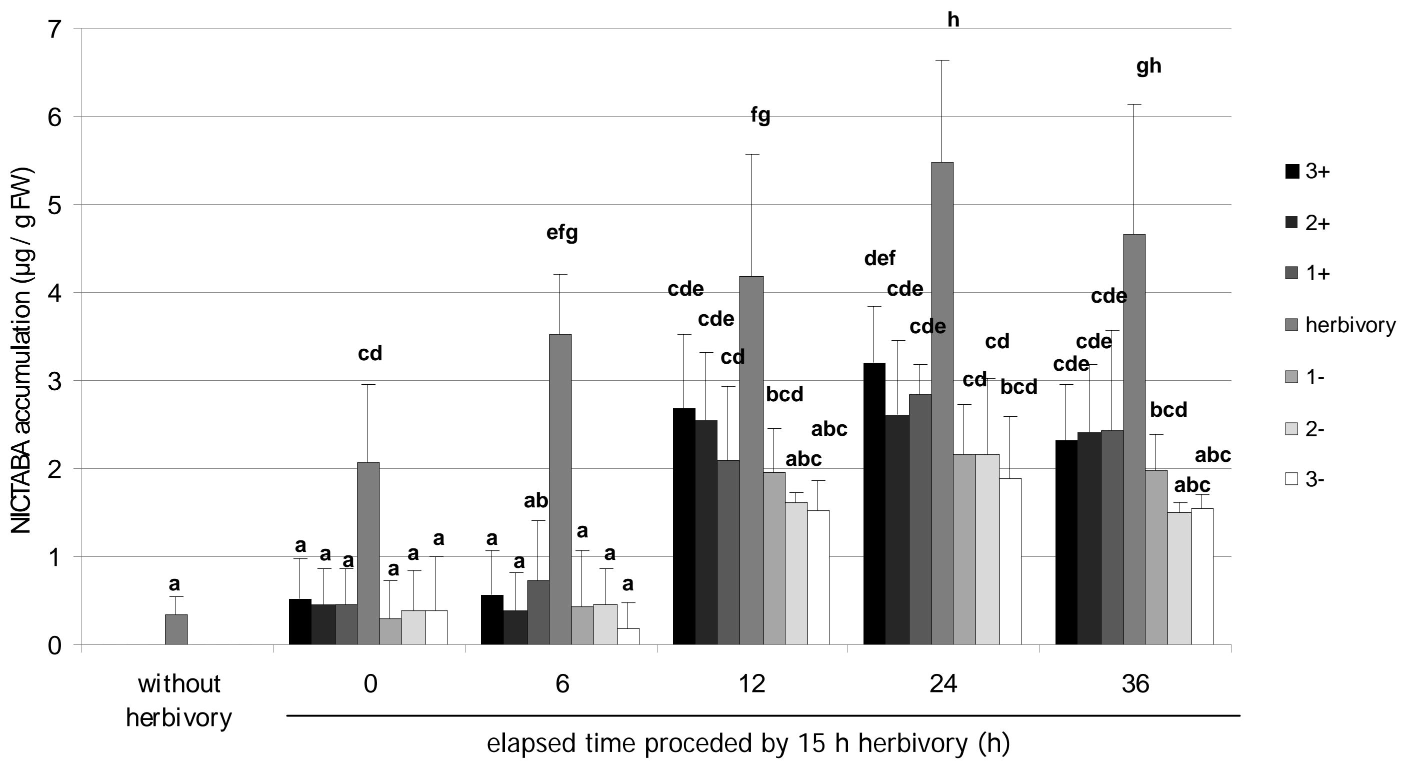

Figure 6