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Jasmonate-Induced Nicotine Formation in Tobacco is Mediated by Tobacco *COI1* and *JAZ* Genes

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Biosynthesis of many plant alkaloids is enhanced by endogenous accumulation and exogenous application of jasmonates, but the general and specific signaling components are not well understood. In *Arabidopsis*, jasmonate-induced ZIM-domain-containing (JAZ) proteins have recently been found to be critical transcriptional repressors linking CORONATINE INSENSTIVE1 (COI1)-mediated jasmonate perception and jasmonate-regulated transcriptional regulation. Insect herbivory on tobacco leaves activates the jasmonate signaling pathway, leading to up-regulation of nicotine biosynthesis genes in roots. We show here that roots of *COI1*-silenced tobacco plants are insensitive to growth inhibition by methyl jasmonate, and do not activate nicotine biosynthesis genes after jasmonate treatment or wounding of leaves. Tobacco JAZ proteins appeared to be rapidly degraded after jasmonate treatment, whereas a C-terminally truncated form lacking the conserved Jas motif did not. When the non-degradable JAZ forms were expressed in tobacco hairy roots, jasmonate induction of nicotine biosynthesis was strongly inhibited. Formation of tobacco alkaloids in jasmonate-elicited tobacco BY-2 cells was also effectively suppressed by the *COI1* RNAi (RNA interference) construct and by the dominant-negative truncated *JAZ* constructs. In addition, jasmonate-mediated induction of nicotine biosynthesis genes was diminished by treatment with a proteasome inhibitor MG132. These results indicate that jasmonate-triggered, COI1-mediated degradation of JAZ repressors activates transcriptional regulation of nicotine biosynthesis genes in tobacco roots.

Keywords: COI1 — Jasmonate — JAZ — Nicotine — Tobacco.

Abbreviations: COI1, CORONATINE INSENSTIVE1; EST, expressed sequence tag; GUS, β-glucuronidase; JA, jasmonate, JAZ; JASMONATE ZIM-domain; MeJA, methyl jasmonate; PI-II, proteinase inhibitor-II; PMT, putrescine *N*-methyltransferase; QPT, quinolinate phosphoribosyltransferase; RNAi, RNA interference; RT–PCR, reverse transcription–PCR; SCF, Skp1–Cullin–F-box; TPI, trypsin proteinase inhibitor.

The nucleotide sequences reported in this paper have been submitted to DDBJ under accession numbers AB433899 (*NtCOI1*), AB433896 (*NtJAZ1*), AB433897 (*NtJAZ2*) and AB433898 (*NtJAZ3*).

Introduction

Jasmonic acid and methyl jasmonate (MeJA), collectively called jasmonates, are ubiquitous and essential plant hormones regulating defense responses against environmental stresses, such as drought, ozone, UV, wounding, and attack by pathogens and insects (Farmer et al. 2003). Jasmonates also play regulatory roles in developmental processes including tuber formation, tendril coiling, flower development and senescence. To accomplish such broad physiological and developmental roles, the jasmonate signaling pathway is intertwined with other hormonal and developmental pathways and alters the expression of a variety of target genes positively or negatively (Ito et al. 2007, Truman et al. 2007).

In *Arabidopsis*, a genetic screening has identified *CORONATINE INSENSTIVE 1* (*COI1*) as a key regulator in the jasmonate signaling pathway (Feys et al. 1994). The COI1 protein is the F-box component of a multiprotein E3-ubiquitin ligase, SCF^{COI1} (Xie et al. 1998), leading to a generally accepted model that ubiquitin-mediated protein degradation via the 26S proteasome is a central mechanism in jasmonate signaling. Recently, JASMONATE ZIM-domain (JAZ) transcriptional repressors emerged as important players of the jasmonate response. The JAZ proteins contain the ZIM motif and the highly conserved Jas motif, and most JAZ members are rapidly induced by MeJA application, insect herbivory and mechanical wounding (Chini et al. 2007, Thines et al. 2007, Yan et al. 2007, Chung et al. 2008). JAZ proteins are degraded in COI1-dependent and 26S proteasome-dependent ways in response to jasmonate treatment of *Arabidopsis* plants (Chini et al. 2007, Thines et al. 2007). Truncated JAZ proteins lacking a C-terminal Jas motif reduce responsiveness to jasmonate in a dominant manner, possibly by stabilizing these JAZ mutant proteins against degradation. Direct physical interaction between COI1 and JAZ1 is stimulated by the jasmonate–isoleucine conjugate (Thines et al. 2007). JAZ3 was also shown to interact directly with MYC2, a key transcriptional activator of jasmonate-regulated genes (Lorenzo et al. 2004, Boter et al. 2006, Dombrecht et al. 2007), and to regulate its activity negatively (Chini et al. 2007). Thus, the JAZ repressors are proposed to link the

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COI1-dependent jasmonate perception with transcriptional regulation of jasmonate-regulated genes (Howe and Jander 2008, Staswick 2008).

Accumulation of a large number of secondary metabolites belonging to structurally diverse groups is well known to be enhanced by exogenous application of jasmonates (Gundlach et al. 1992, van der Fits and Memelink 2000, Howe and Jander 2008). In *Nicotiana* species, insect herbivory and mechanical damage on leaves rapidly induce jasmonate biosynthesis and considerably increase the production and accumulation of trypsin proteinase inhibitors (TPIs) and nicotine (Baldwin et al. 1994). TPIs target insect digestive proteins, whereas nicotine serves as an antifeedant toxin, thereby preventing excessive herbivory by feeding insects (Steppuhn et al. 2004, Steppuhn and Baldwin 2007). Nicotine is synthesized exclusively in the roots, and is then translocated to the leaves via the xylem transpiration system (Dawson 1942, Shoji et al. 2000). Multiple structural genes for nicotine biosynthesis enzymes, including putrescine *N*-methyltransferase (PMT; Hibi et al. 1994) and quinolinate phosphoribosyltransferase (QPT; Sinclair et al. 2000), are transcriptionally activated by exogenous application of jasmonates in tobacco roots and in cultured tobacco cells (Shoji et al. 2000, Goosens et al. 2003, Xu and Timko 2004, Cane et al. 2005). This jasmonate-mediated up-regulation of nicotine biosynthesis genes may involve both a general jasmonate signaling pathway common to higher plants and a distinct signaling component(s) specific to nicotine biosynthesis. A likely candidate for the nicotine-specific component is the tobacco *NIC* regulatory loci, which positively control expression of nicotine biosynthetic genes (Legg 1984, Hibi et al. 1994, Cane et al. 2005).

It is important to point out that approximately 16% of the MeJA-inducible *Arabidopsis* genes do not require COI1 for up-regulation (Devoto et al. 2005). Moreover, 12-oxophytodienoic acid, a cyclopentenone precursor of jasmonates, shows biological activities that are partly shared with jasmonates and induces many defense-related genes in a COI1-independent way (Taki et al. 2005, and references therein). When *COI1* was silenced in *Nicotiana attenuata*, basal and wound-inducible activities of TPIs were effectively abolished, while leaf nicotine levels (which were not wound-inducible in this study) decreased only by approximately 50% (Paschold et al. 2007). When *JAR4* and *JAR6* genes that function in the formation of jasmonate–isoleucine were silenced individually or simultaneously in *N. attenuata* plants, both the basal and the MeJA-induced levels of TPIs were much more strongly reduced than those of nicotine (Wang et al. 2007a, Wang et al. 2007b). These studies using *N. attenuata* may imply that nicotine formation and common defense responses, including TPI induction, are not regulated in exactly the same way by the jasmonate signaling pathway.

In this study, we examined whether the COI1–JAZ signaling pathway is used to regulate biosynthesis of tobacco alkaloids. We isolated tobacco orthologs of *COI1* and three *JAZ* genes, named *NtCOI1* and *NtJAZ1–3*, and demonstrated that they indeed mediate MeJA-induced up-regulation of nicotine biosynthesis genes. The nicotine-specific regulatory branching point in tobacco jasmonate signaling pathways may be placed downstream of the evolutionarily conserved JAZ repressors.

Results

NtCOI1 is required for jasmonate response in tobacco

Since the COI1 F-box protein plays a central role in jasmonate signaling in *Arabidopsis* and tomato (Xie et al. 1998, Li et al. 2004), we isolated a 954 bp cDNA fragment of *Nicotiana tabacum* COI1 ortholog NtCOI1 by reverse transcription–PCR (RT–PCR), and used it to suppress expression of tobacco *COI1* genes by the RNA interference (RNAi)-mediated method. The deduced amino acid sequence of the partial *NtCOI1* cDNA showed 97% identity to the corresponding region of a recently reported COI1 sequence of *N. attenuata* (Paschold et al. 2007). Three *NtCOI1* RNAi lines (CR3, CR17 and CR18) used in this study showed highly reduced levels of *NtCOI1* transcripts in both leaves and roots, as revealed by RT–PCR (Fig. 1A). These *NtCOI1* RNAi seedlings were grown on a medium containing MeJA. Compared with the control wild-type tobacco seedlings, roots of these RNAi seedlings grew significantly better at the jasmonate concentrations tested up to 50 μM (Fig. 1B, C). Growth of aerial organs was also significantly less affected by exogenous application of MeJA in the *COI1*-silenced seedlings. Anther dehiscence in the *COI1*-silenced flowers was impaired, resulting in male sterility (data not shown). Thus, tobacco COI1 is required for the inhibition of root growth by jasmonates.

Silencing of NtCOI1 suppresses jasmonate- and wound-induced biosynthesis of nicotine in tobacco roots

To examine whether nicotine formation is dependent on *NtCOI1*, we induced nicotine biosynthesis by either wounding or jasmonate treatment in *NtCOI1* RNAi plants. Two-month-old tobacco plants grown on soil in a greenhouse were wounded by piercing fully expanded leaves. For jasmonate treatment, 1-month-old plants were grown on agar medium in air-tight plastic pots and were exposed to MeJA vapor emitted from MeJA-soaked cotton balls (Shoji et al. 2000).

First, expression of *PMT* and *QPT*, two structural genes involved in nicotine biosynthesis (Hibi et al. 1994, Sinclair et al. 2000), was examined by RNA gel blot analysis

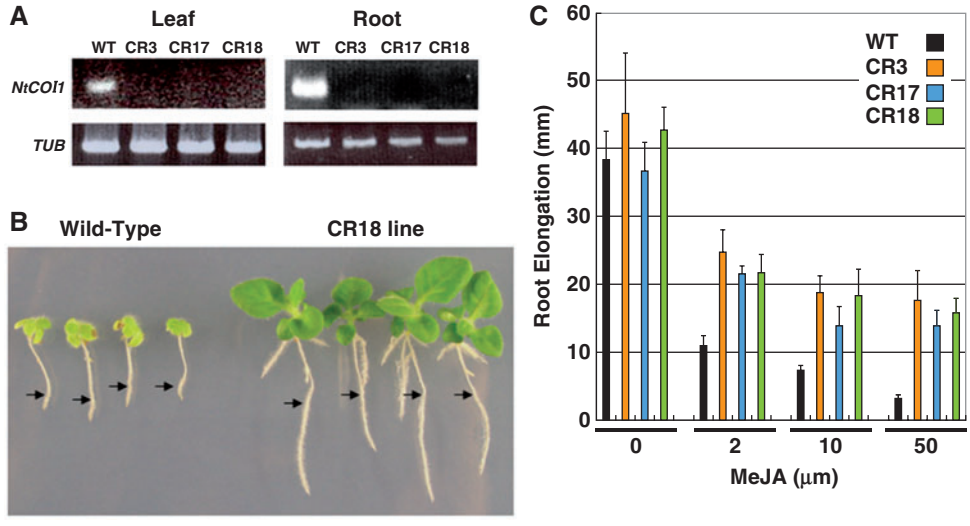

Fig. 1 RNAi-mediated *NtCOI1* silencing in tobacco plants. (A) RT–PCR analysis of *NtCOI1* transcripts in leaves and roots of wild-type (WT) and *NtCOI1* RNAi plants (CR3, CR17 and CR18). α-*Tubulin* (*TUB*) was used as an amplification control. (B and C) Inhibition of root growth by MeJA in wild-type and *NtCOI1* RNAi plants. After growth on normal medium for 7 d, tobacco seedlings were transferred onto the medium containing MeJA. (B) Wild-type and *NtCOI1* RNAi seedlings grown in the presence of MeJA at 50 μM for 8 d. Positions of the root tips at the time of transfer are indicated with arrows. (C) Root growth was quantified 7 d after the transfer to the MeJA-containing medium. Values are means ± SD of more than eight seedlings.

(Fig. 2A, B). When control tobacco plants were treated with MeJA for 24 h, transcript levels of *PMT* and *QPT* were increased approximately 4-fold. This jasmonate-induced up-regulation of *PMT* and *QPT* was completely abolished in the roots of *NtCOI1* RNAi plants (line CR3) (Fig. 2A). Tobacco *QPT* is known to be up-regulated by jasmonate treatment even in the leaves, although the physiological role(s) of this induction in the aerial parts is apparently not related to nicotine biosynthesis (Sinclair et al. 2004). In the leaves of the RNAi plants, jasmonate induction of *QPT* was also diminished considerably. Jasmonate-inducible expression of the *proteinase inhibitor-II* (*PI-II*) gene (Balandin et al. 1985) was similarly inhibited in the leaves of *NtCOI1*-silenced plants (Fig. 2A). Mechanical wounding on control tobacco leaves increased transcript levels of *PMT* and *QPT* approximately 4-fold in the roots after 24 h, whereas neither gene was strongly induced by wounding in the roots of RNAi plants (Fig. 2B). Notably, basal expression levels of *PMT*, *QPT* and *PI-II* without such treatments were indistinguishable between control plants and *NtCOI1* RNAi plants (Fig. 2A, B).

We next analyzed nicotine and other tobacco alkaloids in the leaves of tobacco plants treated with MeJA for 4 d and in the leaves of tobacco plants 6 d after wounding. Jasmonate and wounding treatments increased total alkaloid contents (mostly consisting of nicotine) approximately 2.5- and 2-fold, respectively, in control plants (Fig. 2C, D). However, these treatments did not increase accumulation of tobacco alkaloids in *NtCOI1* RNAi lines (Fig. 2C, D). Interestingly, in the untreated plants, the basal levels of tobacco alkaloids tended to be lower in *NtCOI1*-silenced plants than in control plants. The decrease in basal alkaloid levels was clearly seen in soil-grown and more mature tobacco plants (Fig. 2D). These results indicate that *NtCOI1* is required for wound- and JA-induced up-regulation of nicotine biosynthesis genes in the roots and subsequent increase in alkaloid accumulation in the leaves.

Jasmonate-induced degradation of tobacco JAZ protein

In this study, we obtained three full-length tobacco JAZ cDNAs (*NtJAZ1*, *NtJAZ2* and *NtJAZ3*) by RT–PCR-based cloning using publicly available Solanaceae expressed sequence tag (EST) sequences. NtJAZ1, NtJAZ2 and NtJAZ3 proteins show 40, 36 and 35% overall identities to AtJAZ1, AtJAZ3 and AtJAZ5 of *Arabidopsis thaliana*, respectively. Highly conserved ZIM (Vanholme et al. 2007) and Jas (Yan et al. 2007) motifs are found in these NtJAZ proteins (Fig. 3A, B). In a recently published database of tobacco transcriptional factors (Rushton et al. 2008), 13 JAZ proteins are included in which NtJAZ1 is listed as ZIM15 whereas NtJAZ3 is inaccurately registered as ZIM54 containing a probable unspliced intron. NtJAZ2 is not found in the database. To reveal whether NtJAZs are functional counterparts of AtJAZs, we first examined jasmonate induction profiles of *NtJAZ* genes. When tobacco BY-2 cultured cells were treated with 100 μM MeJA, NtJAZ genes were induced within 1 h and their

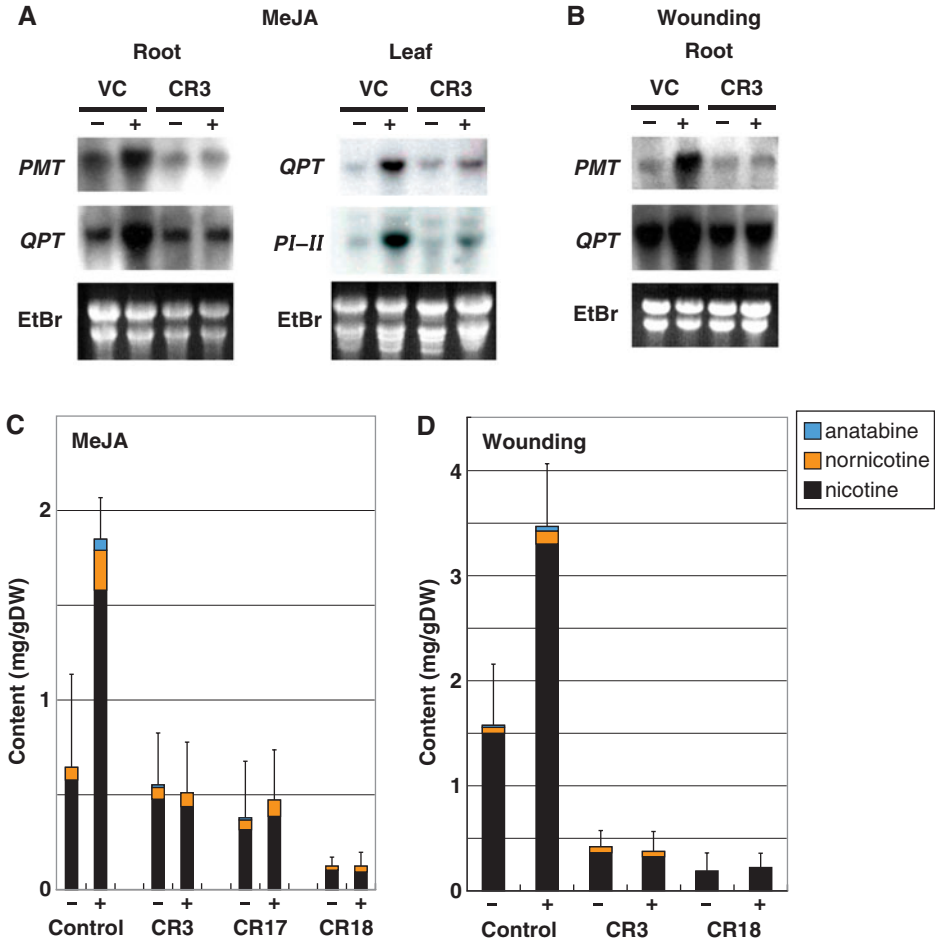

Fig. 2 MeJA- and wound-induced biosynthesis of tobacco alkaloids is suppressed in *NtCOI1* RNAi plants. Plants transformed with an empty vector were used as a control (VC). Treated and untreated plants are indicated with plus and minus signs, respectively. (A) MeJA-induced gene expression. One-month-old plants grown in air-tight containers were exposed to MeJA vapor for 24 h. Total RNAs from leaves and roots were subjected to RNA gel blot analyses. (B) Wound-induced gene expression. Two-month-old plants grown on soil in a greenhouse were wounded on the leaves. After 24 h, roots were harvested for RNA gel blot analysis. (C) MeJA-induced accumulation of tobacco alkaloids in the leaves. Alkaloids were analyzed after 4 d of MeJA treatment. (D) Wound-induced accumulation of tobacco alkaloids in the leaves. Alkaloids were analyzed 6 d after wounding. Data in C and D are means ± SD of more than three samples.

transcript levels declined at 4 h (Fig. 3C). There are differences in their induction profiles; no basal level expression was observed for *NtJAZ1* and *NtJAZ3*, whereas *NtJAZ2* was expressed at a considerable level even without MeJA treatment. Because of their marked induction by MeJA, *NtJAZ1* and *NtJAZ3* were studied further.

We expressed a fusion protein between NtJAZ1 and β-glucuronidase (GUS) in tobacco Bright Yellow-2 (BY-2) cells under the control of the cauliflower mosaic virus (CaMV) 35S promoter to monitor the enzyme activity of GUS as an indicator of the level of NtJAZ1–GUS protein. When cultured tobacco cells from two independent transgenic lines were treated with MeJA at 100 μM, GUS activity decreased by >85% after 1 h (Fig. 3D). Simultaneous addition of the proteasome inhibitor MG132 at 10 μM (Figneiredo-Pereira et al. 1994) effectively abolished the MeJA-induced decrease of GUS activity.

It has been shown that *Arabidopsis* JAZ1 and JAZ3 proteins lacking the C-terminal Jas region were resistant to COI1-dependent degradation after jasmonate treatment, and that dominant, jasmonate-resistant phenotypes were produced (Chini et al. 2007, Thines et al. 2007). Here we fused a similarly truncated version of NtJAZ1 (NtJAZ1ΔC) to GUS to generate NtJAZ1ΔC–GUS. When the tobacco BY-2 cells expressing the NtJAZ1ΔC–GUS protein were challenged with MeJA, the GUS activity did not change (Fig. 3D). These results indicate that NtJAZ1 is degraded immediately after jasmonate application by the 26S proteasome-mediated pathway, and that NtJAZ1 degradation requires its C-terminal region containing the Jas motif.

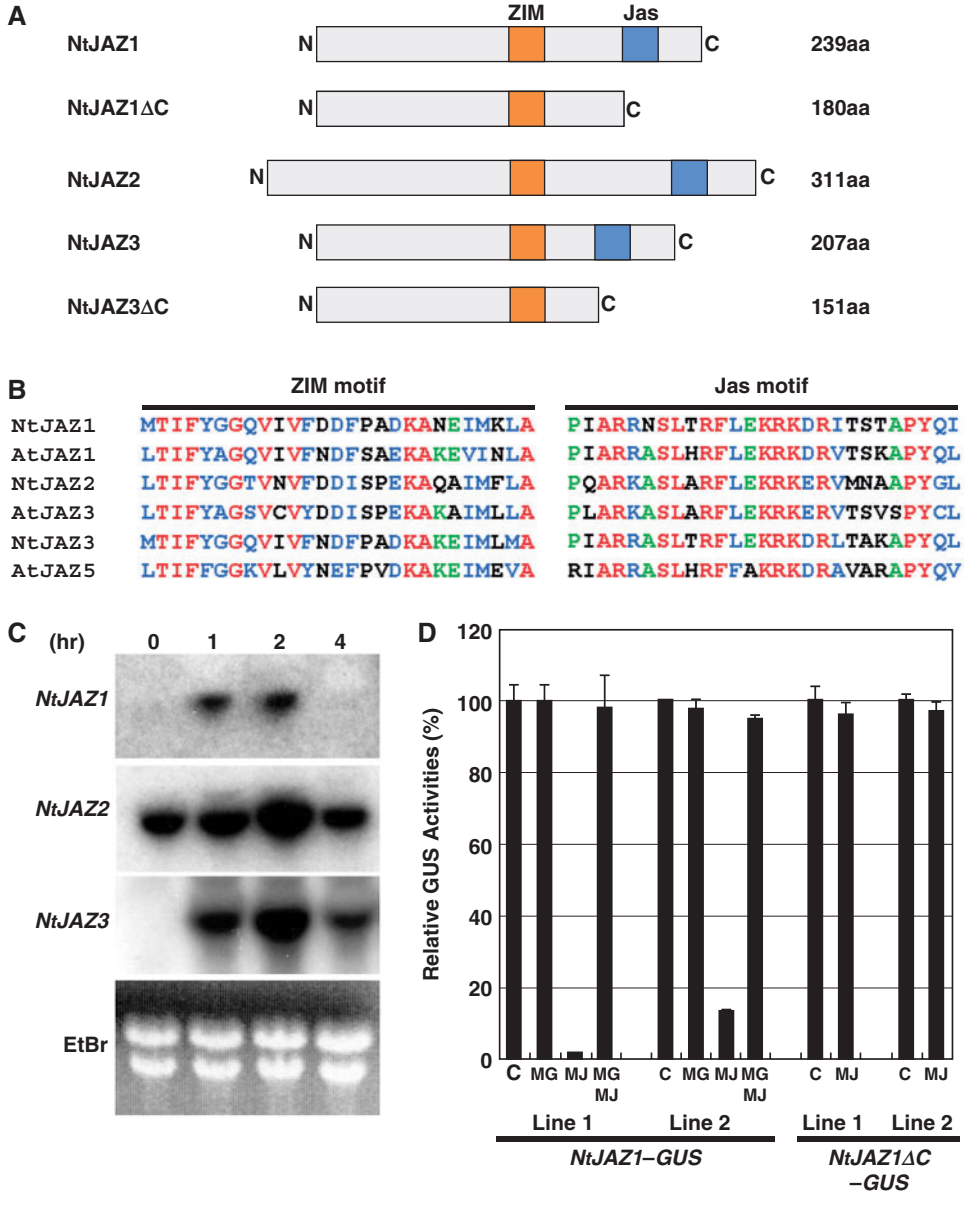

Fig. 3 NtJAZs are induced and degraded after MeJA treatment. (A) NtJAZ1–3 proteins and C-terminal truncated versions. The relative size and position of the conserved ZIM and Jas motifs are shown. (B) Deduced amino acid sequences of the ZIM and Jas motifs in NtJAZs and corresponding *Arabidopsis* homologs (AtJAZ1, AtJAZ3 and AtJAZ5) are aligned. Red, identical residues; blue, conservative substitutions; green, identical in four or five sequences. (C) MeJA-induced expression of *NtJAZ* genes in tobacco BY-2 cells. Tobacco cells were treated with 100 μM MeJA for the indicated periods in an auxin-free medium. (D) MeJA-mediated degradation of NtJAZ1–GUS proteins and its inhibition by a proteasome inhibitor MG132. Tobacco BY-2 cells expressing *NtJAZ1–GUS* or *NtJAZ1ΔC–GUS* were treated with 100 μM MeJA (MJ), 10 μM MG132 (MG) or both for 1 h. GUS activities were quantified and are shown as the percentages relative to the untreated controls (C). Data are means ± SD of four samples.

Dominant-negative NtJAZ forms suppress jasmonate-induced biosynthesis of nicotine in tobacco hairy roots

Three independent tobacco hairy root lines were produced that constitutively expressed NtJAZ1ΔC–GUS or NtJAZ3ΔC which lacks the C-terminal Jas-containing region of NtJAZ3 (Fig. 4A). RNA gel blot analysis showed that basal transcript levels of *PMT* were similar between the vector control roots and the NtJAZ3ΔC roots without MeJA treatment (Fig. 4B). When transgenic tobacco hairy roots were treated with MeJA at 100 μM for 24 h, transcript levels of *PMT* increased considerably in the control roots but not in the NtJAZ3ΔC roots nor in the NtJAZ1ΔC–GUS roots. The GUS activity in the NtJAZ1ΔC–GUS roots did not decrease after MeJA application (data not

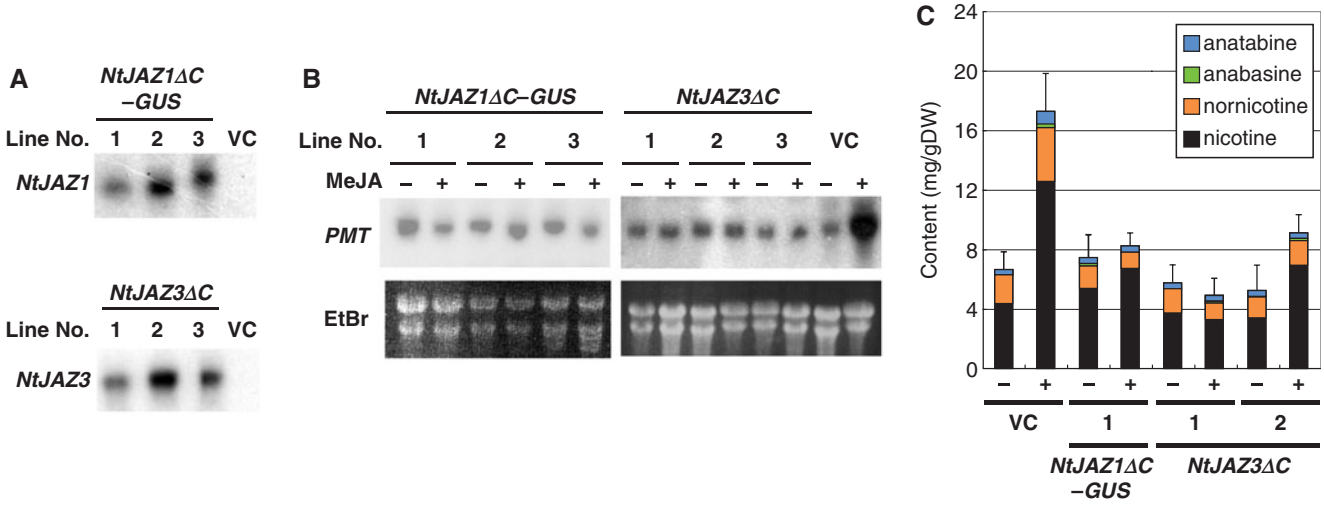

Fig. 4 Dominant-negative forms of NtJAZs suppress MeJA-induced accumulation of tobacco alkaloids in cultured roots. Dominant-negative forms lacking the Jas motifs (NtJAZ1ΔC–GUS and NtJAZ3ΔC) were constitutively expressed in transgenic tobacco hairy roots. Hairy roots transformed with an empty vector were used as a control (VC). (A) RT–PCR analysis of *NtJAZ1ΔC–GUS* and *NtJAZ3ΔC* expression. PCR products were blotted on nylon membranes and then probed with *NtJAZ1* or *NtJAZ3* fragments. (B) RNA gel blot analysis of *PMT* expression. Hairy roots were cultured in the presence (+) or absence (–) of 100 μM MeJA for 24 h. (C) Alkaloid contents. Hairy roots were cultured in the presence (+) or absence (–) of 100 μM MeJA for 3 d. Data are means ± SD of more than three samples.

shown), indicating that NtJAZΔC proteins are resistant to jasmonate-induced degradation. Analysis of tobacco alkaloids (Fig. 4C) showed that MeJA treatment increased the cellular contents of nicotine, nornicotine and anatabine 2.6-fold in control roots, but did not increase them in the NtJAZ1ΔC–GUS roots (line 1) and the NtJAZ3ΔC roots (line 1), or increased them only 1.7-fold in the NtJAZ3ΔC roots (line 2). These results indicate that jasmonate-induced up-regulation of nicotine biosynthesis is mediated by tobacco JAZ repressors.

NtCOI1 and NtJAZ1 mediate MeJA-induced nicotine biosynthesis in tobacco BY-2 cells

Tobacco BY-2 suspension-cultured cells do not produce nicotine when cultured in basic culture medium but can be effectively and rapidly elicited by exogenous jasmonate to synthesize pyridine-type alkaloids, although anatabine rather than nicotine predominates in the jasmonate-elicited tobacco cells (Goossens et al. 2003). To investigate whether *NtCOI1* and *NtJAZ* are also involved in jasmonate-induced synthesis of tobacco alkaloids in this cell culture system, we generated several independent tobacco BY-2 cell lines in which either *NtCOI1* was silenced by RNAi or the *NtJAZ1ΔC–GUS* transgene was stably expressed (Fig. 5A). Three representative cell lines for each construct were treated with MeJA at 100 μM; RNA gel blot analysis was done after 1 d, and tobacco alkaloids were analyzed after 3 d. Strong expression of *PMT* was observed in MeJA-treated vector control cells, whereas PMT was not induced or induced only weakly in the *NtCOI1-RNAi* cells and the *NtJAZ1ΔC–GUS* cells (Fig. 5B). Synthesis of tobacco alkaloids (mainly anatabine, followed by nicotine and nornicotine) was effectively induced by MeJA application in the control cells, but alkaloid formation in the *NtCOI1-RNAi* cells and the *NtJAZ1ΔC–GUS* cells was severely reduced to approximately 5–15% of the control levels (Fig. 5C). These results indicate that *NtCOI1* and *NtJAZ* are required for jasmonate-induced biosynthesis of pyridine alkaloids in tobacco BY-2 cells.

Proteasome inhibitor suppresses MeJA-induced gene expression involved in nicotine biosynthesis

The above results are consistent with a model in which jasmonate-triggered COI1-dependent degradation of JAZ repressors activates structural genes for nicotine biosynthesis. To provide further support for this model, we treated tobacco BY-2 cells with MeJA at 100 μM and/or a proteasome inhibitor MG132 at 10 μM for 6 h. MG132 was applied to the tobacco cells 30 min prior to the MeJA addition. The general wound- and jasmonate-inducible gene *PI-II* was expressed at a basal level before MeJA treatment (Fig. 6). MeJA treatment strongly induced expression of *PMT* and *QPT* and markedly up-regulated *PI-II* expression. These effects of MeJA were effectively suppressed by MG132, while MG132 alone did not affect expression levels of these genes in the absence of MeJA (Fig. 6). Therefore, a MG132-sensitive proteasome pathway is necessary for MeJA-induced expression of *PMT* and *QPT* in tobacco BY-2 cells.

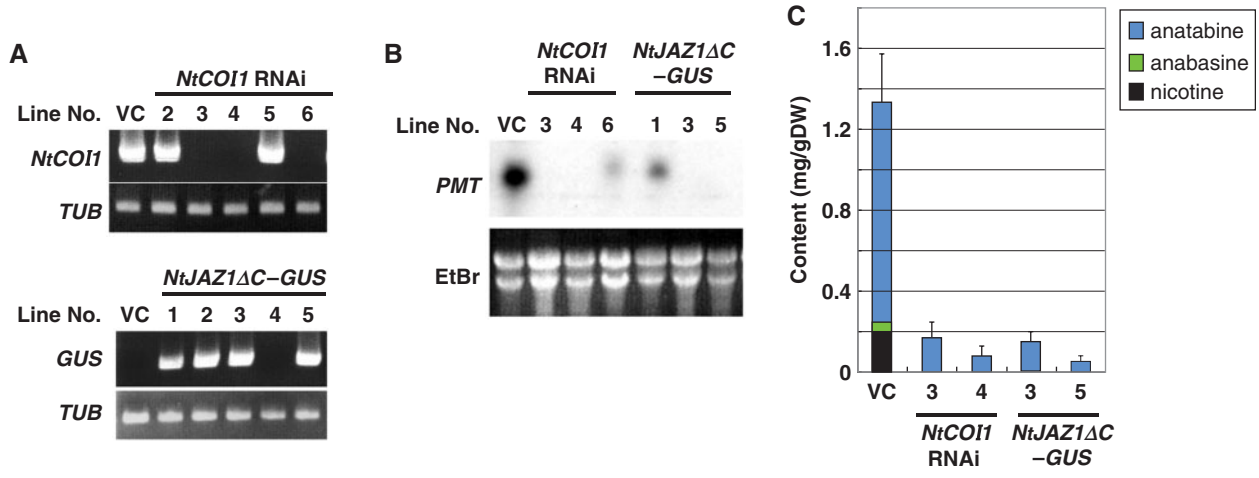

Fig. 5 Requirement of the COI1–JAZ pathway for MeJA-induced biosynthesis of tobacco alkaloids in cultured BY-2 cells. Tobacco cell lines transformed with *NtCOI1*, *NtJAZ1ΔC–GUS* or an empty vector (VC) were analyzed. Cultured tobacco cells were treated with 100 μM MeJA in an auxin-free medium to induce alkaloid formation. (A) RT–PCR analyses of *NtCOI1* and *NtJAZ1ΔC–GUS* in corresponding lines. α-*Tubulin* (*TUB*) was used as an amplification control. (B) RNA gel blot analysis of *PMT* expression. (C) Alkaloid contents. Data are means ± SD of more than three samples.

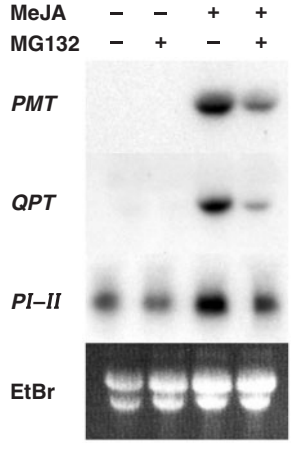

Fig. 6 Proteasome inhibitor suppresses MeJA-induced gene induction of nicotine biosynthesis genes. Tobacco BY-2 cells were treated with 100 μM MeJA, 10 μM MG132 or both for 8 h in an auxin-free medium. Transcript levels of *PMT*, *QPT* and *PI-II* were analyzed by RNA gel blots.

Discussion

Conserved COI1–JAZ signaling pathway in tobacco

The F-box protein COI1 of *A. thaliana* has been extensively studied and is firmly established to play a central role in jasmonate signaling by forming the Skp1–Cullin–F-box (SCF) complexes in the ubiquitination pathway (Howe and Jander 2008). Studies on jasmonate-insensitive mutants of tomato show that the tomato homolog of COI1 is also essential for jasmonate signaling in tomato (Li et al. 2004, Li et al. 2006). The soybean homolog of COI1 complemented the *Arabidopsis coi1* mutant, and assembled into the SCF complexes, indicating the conserved function of soybean COI1 (Wang et al. 2005). In *N. attenuata* (Paschold et al. 2007) and *N. tabacum* (this study), transgenic suppression of *Nicotiana COI1* homologs resulted in jasmonate insensitivity of root growth, impaired anther dehiscence and down-regulated jasmonate-responsive genes. These studies suggest that the COI1-related F-box protein is an essential conserved component in a generic jasmonate pathway in diverse dicotyledonous plants.

Recent characterization of *Arabidopsis* JAZ proteins implicates that the jasmonoyl–isoleucine conjugate promotes SCF^{COI1} interaction with JAZ transcriptional repressors, leading to their ubiquitination and degradation by the 26S proteasome (Staswick 2008). Apart from the conserved JIM and Jas motifs, *Arabidopsis* JAZ proteins diverge considerably, ranging from 131 to 352 amino acids in length, and the relative position of ZIM and Jas varies. Other plants also contain JAZ-like proteins, which may function in a similar way. Indeed, tomato COI1 and a tomato JAZ interacted in a jasmonoyl–isoleucine-dependent manner in yeast and in a cell-free system (Thines et al. 2007). In this study, we demonstrated that tobacco JAZ proteins are induced by MeJA application, are degraded after MeJA application in a proteasome-dependent manner and are converted to dominant-negative forms by deletion of the Jas motif. These properties of tobacco JAZ proteins are consistent with those of *Arabidopsis* JAZ proteins (Chini et al. 2007, Thines et al. 2007, Yan et al. 2007, Chung et al. 2008), indicating that the SCF^{COI1}–JAZ pathway is also conserved for jasmonate signaling in tobacco.

Requirement of the COI1–JAZ pathway for nicotine biosynthesis

Biosynthesis of nicotine and related pyridine alkaloids is under strict spatial and temporal control in tobacco plants (Katoh et al. 2005). Specific transcriptional regulation of structural genes for nicotine biosynthesis must have occurred concomitantly with or just after molecular evolution of the structural genes in *Nicotiana* species. Wound- and jasmonate-inducible components of the regulatory network might utilize the common signaling block of jasmonate responses, which may have a downstream nicotine-specific regulator(s). In this sense, it is quite important to examine whether the established COI1–JAZ pathway is used to control biosynthesis of tobacco alkaloids. Previously, suppression of *COI1* or *JAR4/6* (encoding enzymes that synthesize amino acid conjugates with jasmonic acid) in *N. attenuata* resulted in compromised accumulation of nicotine, but the effects on nicotine synthesis were much lower than those on other jasmonate-elicited responses, including induction of TPIs (Paschold et al. 2007, Wang et al. 2007a, Wang et al. 2007b). Our studies using three different tobacco systems (plants, hairy roots and cultured cells) clearly showed that jasmonate-induced synthesis of tobacco alkaloids strongly depends on the COI1–JAZ signaling pathway. Interestingly, interruption of the COI1–JAZ pathway affected basal (uninduced) levels of nicotine in plant roots and cultured hairy roots much less strongly than jasmonate-induced nicotine synthesis. There may be an additional regulatory pathway that activates nicotine biosynthesis in the tobacco roots.

Since tobacco *NIC* loci specifically control expression of structural genes for nicotine biosynthesis and transport (Hibi et al. 1994, Cane et al. 2005, Katoh et al. 2005, Katoh et al. 2007, our unpublished results), it is tempting to speculate on the relationship between *NIC* and the COI1–JAZ pathway. In a simple model, NIC proteins are transcription factors that positively regulate expression of nicotine biosynthesis genes, and are normally inactivated by interaction with JAZ repressors. As proposed for the interaction of the MYC2 transcription factor with JAZ proteins in *Arabidopsis* (Chini et al. 2007), jasmonate signaling promotes degradation of tobacco JAZ repressors, leading to the activation of NIC regulators. Molecular cloning of *NIC* genes is highly anticipated to test whether this simple scenario holds true.

Materials and Methods

Plant materials and transformation

Plants and hairy roots of tobacco (*N. tabacum* cv. Petit Hanava line SR1 and cv. Burley21) were grown and treated with MeJA as described (Shoji et al. 2000). For wounding treatment, 2-month-old tobacco plants grown on soil in a greenhouse were wounded by piercing a hole (1 cm diamater) in the middle portions of five fully expanded leaves. Roots and leaves were harvested for RNA gel blot analysis after 1 d, and for alkaloid analysis after 6 d. Transgenic hairy roots were generated by infecting leaf discs with *Agrobacterium rhizogenes* strain ATCC 15834 as described (Kanegae et al. 1994). After selection and disinfection by culturing several times on solid medium containing 50 mg l^{-1} kanamycin and 250 mg l^{-1} claforan, hairy roots were subcultured in liquid MS medium every 2 weeks.

Tobacco BY-2 cells were subcultured in liquid MS medium supplemented with 20 mg l^{-1} KH_2PO_4, 0.5 g l^{-1} MES and 0.2 mg l^{-1} 2,4-D every week. Transgenic tobacco BY-2 cells were generated with *Agrobacterium tumefaciens* strain EHA105 as described (An 1985). Four-day-old BY-2 cells were first rinsed to remove 2,4-D, and then transferred to fresh auxin-free MS medium supplemented with the indicated chemicals. When treated with both MeJA and MG132, cultured cells were pre-treated with MG132 for 30 min prior to MeJA addition.

DNA cloning and construction

To isolate tobacco *COI1* orthologs, a pair of degenerate PCR primers was designed to anneal to the sequences conserved among the COI1 proteins of *Arabidopsis* (Xie et al. 1998), rice, maize and tomato (Li et al. 2004). PCR primers for tobacco *JAZ* genes were designed based on several Solanaceae EST sequences with limited homologies to *Arabidopsis JAZ1*, *JAZ3* and *JAZ5* genes (Staswick 2008). We performed RT–PCR using total RNA from tobacco leaves (cv. Burley21) for *NtCOI1*, and from tobacco BY-2 cells treated with 100 μM MeJA for 2 h for *NtJAZs* with pairs of primers (*NtCOI1*, 5′-YTIAAYTAYATGACIGA and 5′-GCIC KYTCISWRAARCARCA; *NtJAZ1*, 5′-ATGGGGTCATCGGA GATTGTAGATTCC and 5′-CTAAAAGAACTGCTCAGTTTT CACTGG; *NtJAZ2*, 5′-ATGGAGAGATTTTATGGGACTG and 5′-CTAGGTCTCCTTACCGGCTATC; *NtJAZ3*, 5′-ATGG CATCGGAGATTGTGG and 5′-CTAGAATTGCTCAGCTTT CACTGG). A partial cDNA clone was obtained for *NtCOI1* in this study. PCR products were cloned into pGEM-T Easy by a TA cloning system (Promega, Madison, WI, USA) and sequenced.

For RNAi-mediated suppression of tobacco *COI1*, a portion of the *NtCOI1* cDNA (corresponding to amino acid residues 230–370 of *Arabidopsis* COI1) was first subcloned into pHANNIBAL vector (Wesley et al. 2001) and then inserted into binary vector pBI121 (Clontech, Moutain View, CA, USA). For construction of pBI-NtJAZ1-GUS and pBI-NtJAZ1ΔC-GUS, a full-length coding region and a portion of the *NtJAZ1* (amino acid residues 1–180) were amplified by PCR and inserted between the *Xba*I and *Bam*HI sites in pBI101 (Clontech). pBI-NtJAZ3ΔC was constructed after a portion of the *NtJAZ3* (amino acid residues 1–151) was amplified by PCR and cloned into the *Xba*I and *Sac*I sites of pBI121. A stop codon was introduced or removed in the reverse primers, depending on the constructs. A high-fidelity KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was used for PCR amplification, and PCR products were sequenced to verify no unintentional mutations.

Alkaloid analysis

Samples (50 mg dry weight) were lypophilized, homogenized and soaked in 4 ml of 0.1 N H_2SO_4. The homogenate was sonicated for 60 min and centrifuged at 2,000 × *g* for 15 min. The supernatant was neutralized by adding 0.4 ml of 25% NH_4OH. The mixture (1 ml) was loaded onto an Extrelut-1 column (Merck) and eluted with 6 ml of chloroform. The elutant was dried at 37°C. The dry residues were dissolved in ethanol containing 0.1% dodecane as an internal standard, and analyzed by gas–liquid chromatography

(GC-14B; Shimadzu, Kyoto, Japan) equipped with an Rtx-5 Amine capillary column (Shimadzu). The column temperature was held at 100°C for 10 min, then increased from 100 to 250°C in 35 min. Each peak was assigned and calibrated with authentic standards.

RNA gel blot and RT–PCR analyses

Total RNA was isolated from frozen tissues using an RNeasy kit (Qiagen, Valencia, CA, USA), separated by electrophoresis on 1.2% formaldehyde agarose gels, and blotted onto a Hybond-N+ nylon membrane (Amersham). Equal loading on each lane was confirmed by staining the gels with ethidium bromide before blotting. The blots were hybridized in 50% (v/v) formamide, 5× SSPE, 2× Denhardt's solution and 0.1% (w/v) SDS at 42°C for 16 h with ^{32}P-labeled DNA probes prepared by using a Bcabest labeling kit (TAKARA SHUZO CO. LTD., Kyoto, Japan). Washing was performed three times in 0.2× SSPE and 0.1% (w/v)SDS at 65°C, and then the blots were exposed to X-ray films for image acquisition. Tobacco cDNA fragments of *PMT* (Hibi et al. 1994), *QPT* (Sinclair et al. 2000) and *PI-II* (Balandin et al. 1995) were used for hybridization probes.

First-strand cDNA was synthesized from total RNA by SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Aliquots of the first-strand cDNA were amplified with primers (*NtCOI1*, 5′-CCCANAAGCATCCATCTCAC and 5′-GAAGATCTTGAATTGATGGC; *α-tubulin*; 5′-AGTTGGAGGAGGTGATGATG and 5′-TATGTGGGTCGCTCAATGTC, *GUS*, 5′-TATTGCCAACGAACCGGATACCCGT and 5′-ACGATGGTGCGCCAGGAGAGTTGTT; *NtJAZ1*, 5′-ATGGGGTCATCGGAGATTGTAGATTCC and 5′-CGGGATCCTAAATCAGCAACACTGGATTGT; *NtJAZ3*, 5′-ATGGCATCATCGGAGATTGTGG and 5′-CTCGTCTCGAGCTCCTATAGATCTCCAGAAATAATAGG) for 3 min at 94°C, followed by cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, and finally a 10 min incubation at 72°C. The amplified products were separated through 1% agarose gel, and were stained with ethidium bromide or blotted onto a Hybond-N+ nylon membrane for DNA blot analysis. The DNA blot analysis was done as described (Sambrook et al. 1989) using ^{32}P-labeled *NtJAZ1* or *NtJAZ3* cDNA probes prepared by a Bcabest labeling kit. To avoid saturated PCR amplification, the amounts of template cDNA and PCR cycle numbers were adjusted in all cases.

Fluorometric GUS assays

GUS activity was quantified fluorometrically according to Jefferson (1987) with some modifications. Tobacco cells were homogenized in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and 5 mM dithiothreitol. After centrifugation at 15,000 × *g* for 30 min, the supernatants were desalted with a NAP-5 column (Amersham). The desalted enzyme solution was incubated with 0.05 mM 4-methylumbelliferyl-β-D-glucuronide at 30°C. The amount of 4-methylumbelliferone formed was measured using a fluorescence spectrometer (F-4500; Hitachi, Ibaraki, Japan). Protein concentration was determined with a Coomassie protein assay reagent (Pierce).

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