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# High levels of jasmonic acid antagonize the biosynthesis of gibberellins and inhibit the growth of *Nicotiana attenuata* stems

**Maria Heinrich[1,‡], Christian Hettenhausen[1,‡], Theo Lange[2], Hendrik Wünsche[1], Jingjing Fang[3], Ian T. Baldwin[1] and Jianqiang Wu[1,‡,*]**

[1]*Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, D-07745 Jena, Germany,*

[2]*Institute of Plant Biology, Department of Plant Physiology and Biochemistry, Technical University of Braunschweig, Braunschweig D-38106 Germany, and*

[3]*Research Group Biosynthesis/NMR, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, D-07745 Jena, Germany*

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*For correspondence (e-mail jwu@ice.mpg.de).
The authors declare no conflict of interest.
‡Present address: Key Laboratory of Economic Plants and Biotechnology, Kunming Institute of Botany, Chinese Academy of Sciences, Lanhei Road 132, 650201 Kunming, China.

## SUMMARY

**Hormones play pivotal roles in regulating plant development, growth, and stress responses, and cross-talk among different hormones fine-tunes various aspects of plant physiology. Jasmonic acid (JA) is important for plant defense against herbivores and necrotic fungi and also regulates flower development; in addition, Arabidopsis mutants over-producing JA usually have stunted stems and wound-induced jasmonates suppress Arabidopsis growth, suggesting that JA is also involved in stem elongation. Gibberellins (GAs) promote stem and leaf growth and modulate seed germination, flowering time, and the development of flowers, fruits, and seeds. However, little is known about the interaction between the JA and GA pathways. Two calcium-dependent protein kinases, CDPK4 and CDPK5, are important suppressors of JA accumulation in a wild tobacco species, *Nicotiana attenuata*. The stems of *N. attenuata* silenced in *CDPK4* and *CDPK5* (irCDPK4/5 plants) had dramatically increased levels of JA and exhibited stunted elongation and had very high contents of secondary metabolites. Genetic analysis indicated that the high JA levels in irCDPK4/5 stems accounted for the suppressed stem elongation and the accumulation of secondary metabolites. Supplementation of $GA_3$ to irCDPK4/5 plants largely restored normal stem growth to wild-type levels. Measures of GA levels indicated that over-accumulation of JA in irCDPK4/5 stems inhibited the biosynthesis of GAs. Finally, we show that JA antagonizes GA biosynthesis by strongly inhibiting the transcript accumulation of *GA20ox* and possibly *GA13ox*, the key genes in GA production, demonstrating that high JA levels antagonize GA biosynthesis in stems.**

**Keywords: jasmonic acid, gibberellin, phytohormone interaction, stem elongation, gibberellin biosynthesis, *Nicotiana attenuata*.**

## INTRODUCTION

Hormone signaling pathways play critical roles in the physiology of multicellular organisms. In plants, auxins, gibberellins (GAs), jasmonic acid (JA), ethylene, brassinosteroids (BRs), abscisic acid (ABA), salicylic acid (SA), cytokinins (CKs), and strigolactones are essential for plant development, growth, and interaction with environmental factors. Among these, auxins, GAs, JA, ethylene, BRs, CKs, and strigolactones have been intensively studied for their roles in plant development (Grant and Jones, 2009; Wolters and Jurgens, 2009; Lau and Deng, 2010; Depuydt and Hardtke, 2011; Krouk *et al.*, 2011), and JA, ethylene, SA, and ABA are well known for their functions in plant resistance to abiotic and biotic stresses (Maffei *et al.*, 2007; Browse, 2009; Grant and Jones, 2009; Wu and Baldwin, 2010).

Given their strong influence on plant physiology, the biosynthesis, degradation, and the components of these phytohormone signaling networks are tightly regulated. Mutants or genetically modified plants with altered levels of hormones or defects in hormone signaling generally exhibit abnormal growth, development, and/or resistance to environmental stresses.

Jasmonic acid has been intensively studied for its important role in regulating plant responses to wounding and herbivore feeding (Wasternack, 2007; Wu and Baldwin, 2010). Chloroplast membrane lipids are converted to 12-oxo-phytodienoic acid (OPDA) in the chloroplast, and OPDA is further transported to the peroxisome where it forms JA (Wasternack, 2007). Jasmonic acid is converted to a JA-isoleucine conjugate (JA-Ile) in the cytoplasm, and JA-Ile binds to an F-box protein, the receptor of JA-Ile, CORONATINE INSENSITIVE 1 (COI1; Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2009). Binding of JA-Ile to COI1 leads to degradation of JASMONATE ZIM-DOMAIN (JAZ) proteins, which are key transcription repressors of JA-dependent responses (Boter *et al.*, 2004; Chini *et al.*, 2007). Plants having defects in JA production or signaling not only have dramatically compromised defense against herbivores and necrotrophic fungi (Vijayan *et al.*, 1998; Paschold *et al.*, 2007) but also exhibit male sterility, although they have relatively normal vegetative growth.

Despite its importance in defense and flower development, levels of JA are highly regulated to balance growth and defense, as elevated JA enhances defense but also causes reduced growth. Baldwin (1998) found that exogenously applied jasmonates highly compromised plant fitness but enhanced defense. Similarly, Arabidopsis plants producing excessive JA have reduced size but show increased resistance to fungi and insect herbivores (Bonaventure *et al.*, 2007; Hyun *et al.*, 2008), and when Arabidopsis is repeatedly wounded, its growth is also highly stunted, due to the wound-induced accumulation of JA (Zhang and Turner, 2008). However, the underlying mechanism by which elevated JA levels inhibit plant growth is unclear.

Gibberellins play critical roles in seed germination, stem and leaf elongation, and the development of flowers, fruits, and seeds. Seeds of several plant species deficient in GAs cannot germinate without exogenously applied GAs, and plants with low levels of GAs have reduced size and fertility (Lange and Lange, 2006). The biosynthesis and signaling of GAs have been studied intensively. Gibberellins are diterpene compounds synthesized from geranylgeranyl diphosphate through a complex pathway (Yamaguchi, 2008). DELLA proteins are the master suppressors for GA-induced responses and thus control many aspects of development, growth, and even stress resistance (Sun, 2010, 2011). Bioactive GAs bind to the receptor GIBBERELLIN INSENSITIVE DWARF 1 (GID1; Ueguchi-Tanaka *et al.*, 2005) and enhance the degradation of DELLA proteins through an F-box protein SLEEPY1 (SLY1)-mediated ubiquitin-26S-proteasome pathway (Griffiths *et al.*, 2006; Ueguchi-Tanaka *et al.*, 2007).

A growing body of evidence has indicated that plant hormones not only have specific functions but also interact with each other (Grant and Jones, 2009; Depuydt and Hardtke, 2011). For example, SA inhibits JA signaling through an NPR1-mediated pathway (Spoel *et al.*, 2003) and the gaseous hormone ethylene is also involved in the suppressive effect of SA on JA signaling (Diezel *et al.*, 2009; Leon-Reyes *et al.*, 2009). Auxin interacts with CKs, ethylene, and BRs to regulate plant development (Swarup *et al.*, 2002). Cross-talk between GAs and other hormones has also been demonstrated. It is well known that GAs promote seed germination, while ABA antagonizes GAs and thus inhibits germination (Finkelstein *et al.*, 2008). The GA and ABA signaling pathways may converge on DELLAs to modulate root development, both positively and negatively (Achard *et al.*, 2006). Furthermore, auxin interacts positively with the GA pathway by promoting the degradation of DELLAs or the biosynthesis of GAs (Fu and Harberd, 2003).

Some evidence has shown that JA and GA pathways also interact. An Arabidopsis quadruple DELLA mutant is partially insensitive to gene induction by methyl jasmonate, while the *gai* (constitutively active dominant DELLA) mutant is hypersensitive to JA induction (Navarro *et al.*, 2008), since DELLAs affect jasmonate signaling by competitive binding to JAZs (Hou *et al.*, 2010). A recent study also indicated that Arabidopsis and rice with defects in *COI1* are hypersensitive to GAs, and JA negatively regulates GA responses by affecting the levels of DELLAs (Yang *et al.*, 2012b).

The biosynthesis and signaling of JA have been intensively studied for their functions in plant resistance to insect herbivores in a wild tobacco plant, *Nicotiana attenuata* (Halitschke and Baldwin, 2003; Paschold *et al.*, 2007; Wang *et al.*, 2008). Several genes were found to have a strong impact on JA accumulation, such as two mitogen-activated protein kinases (MAPKs), *SALICYLIC ACID-INDUCED PROTEIN KINASE* (*SIPK*) and *WOUND-INDUCED PROTEIN KINASE* (*WIPK*) (Wu *et al.*, 2007), *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*; Yang *et al.*, 2011), *S-NITROSOGLUTATHIONE REDUCTASE* (*GSNOR*; Wünsche *et al.*, 2011a), and *SUPPRESSOR OF G-TWO ALLELE OF SKP1* (*SGT1*; Meldau *et al.*, 2011). In addition, *N. attenuata* silenced in two *CALCIUM-DEPENDENT PROTEIN KINASE*s (*CDPK*s), *CDPK4* and *CDPK5* (hereafter, irCDPK4/5 plants), exhibits dramatically increased JA levels after wounding or insect feeding, and shows stunted stem elongation and prematurely aborted flower buds and flowers (Yang *et al.*, 2012a).

Here we show that compared with those of the wild type (WT), stems of irCDPK4/5 plants contained dramatically

increased levels of JA, and our genetic analysis indicated that these high JA contents accounted for the stunted stem growth of irCDPK4/5. Exogenously applying bioactive $GA_3$ to irCDPK4/5 could largely restore normal stem growth, and importantly, quantification of GAs confirmed that irCDPK4/5 plants were highly deficient in GAs. We further demonstrate that elevated JA levels suppressed the transcript levels of several GA biosynthetic genes, among which *GA20ox* and possibly *GA13ox* levels were drastically decreased. We propose that increased levels of JA antagonize the biosynthesis of GAs by repressing the transcription of the GA biosynthetic genes *GA13ox* and *GA20ox*.

## RESULTS

### irCDPK4/5 plants are highly stunted in stem elongation

Previously it has been shown that *N. attenuata* silenced in *CDPK4* and *CDPK5* has darker green leaves, highly decreased fertility, and stunted stem growth compared with WT. These phenotypes were observed in almost all of the 10 independently transformed lines produced as well as in irCDPK4/5-1 and irCDPK4/5-2, which were studied in detail for their defense against herbivores (Yang *et al.*, 2012a). In this study we focused on a single line, irCDPK4/5-1 (hereafter irCDPK4/5 plants), which was phenotypically indistinguishable from the other lines studied. To quantitatively examine plant growth, WT and irCDPK4/5 plants were grown concurrently under the glasshouse conditions optimized for *N. attenuata* and their growth parameters were measured over time. Twenty-nine days after germination, when the stems of WT and irCDPK4/5 slightly elongated, no large differences were found between the lengths of WT and irCDPK4/5 stems (both about 2.5 cm); however, starting from 31 days after germination, stems of irCDPK4/5 appeared to be more than 50% shorter than those of WT plants (Figure 1a and Figure S1a in Supporting Information). The diameters of the irCDPK4/5 rosette were similar to those of WT until 26 days after germination, but thereafter irCDPK4/5 rosette leaves were slightly shorter (up to 15%; Figures 1b and S1b).

Microscopic analysis was performed to determine if the stunted stems of irCDPK4/5 plants were correlated with decreased cell expansion. Indeed, compared with those of WT stems, the average length of the pith cells of irCDPK4/5 plants was about 40% reduced, as seen in the longitudinal sections, and the diameters of the pith cells were 35% smaller in the cross sections (Figures 1c, d and S2).

To further analyze the function of CDPK4 and CDPK5 in stem development, promoters of *CDPK4* and *CDPK5* were fused to the *β-glucuronidase* (*uidA* or 'GUS') reporter gene and transformed into *N. attenuata* to create CDPK4Pro:GUS and CDPK5Pro:GUS plants, respectively. We found that *CDPK4* was expressed in xylem, phloem, and cambium cells and *CDPK5* was expressed in xylem and cambium cells (Figure 2), consistent with a function of CDPK4 and CDPK5 in the stems of *N. attenuata*.

### Silencing *CDPK4* and *CDPK5* dramatically increases JA and secondary metabolite contents of *N. attenuata* stems

After wounding and simulated herbivore feeding, leaves of irCDPK4/5 plants produce more than five- and twofold greater levels of JA, respectively, than do WT plants and have highly elevated defense levels, making them highly resistant to the insect herbivore *Manduca sexta* (Yang *et al.*, 2012a). We speculated that similar to the leaves, the stems of irCDPK4/5 may also accumulate high contents of

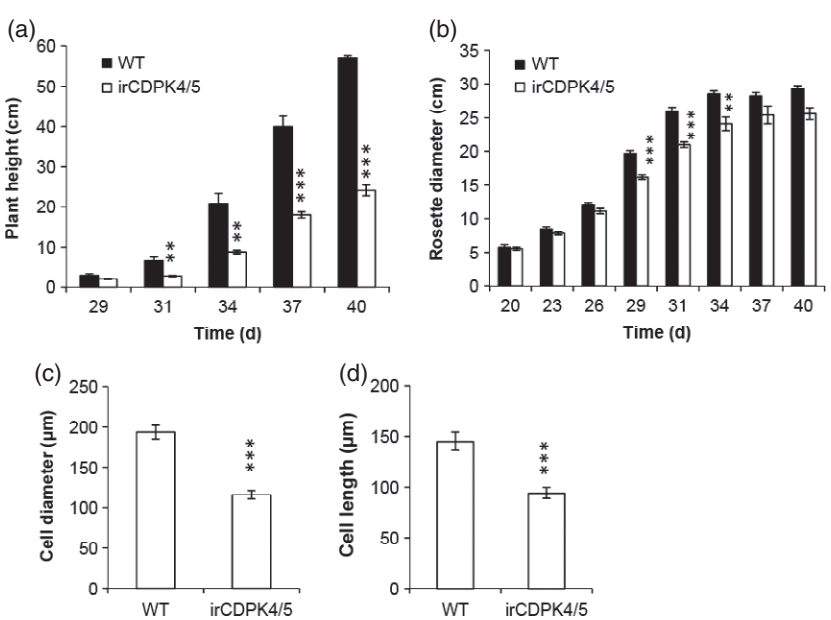


**Figure 1.** Silencing *CDPK4/5* results in stunted stem growth in *Nicotiana attenuata*.
Wild-type (WT) and irCDPK4/5 plants were cultivated concurrently. Their heights (a) and rosette diameters (b) (mean ± SE, $n = 15$) were recorded through 40 days after germination. Sizes of pith cell (about 75 cells from five plants for each type of plant) were measured in cross (c) and longitudinal sections (d) of WT and irCDPK4/5 stems from 40-day-old plants. Asterisks indicate significant differences between WT and irCDPK4/5 plants (*t*-test; $^{**}P < 0.01$; $^{***}P < 0.001$).

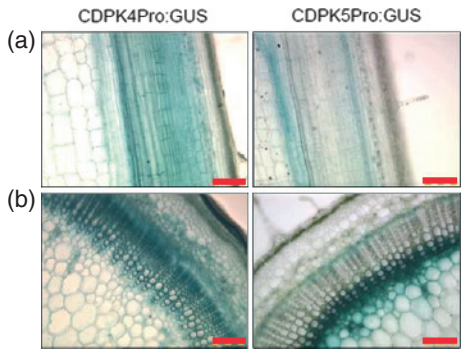


**Figure 2.** The expression of *CDPK4* and *CDPK5* in *Nicotiana attenuata* stems. The longitudinal (a) and cross sections (b) of stems of CDPK4Pro:GUS and CDPK5Pro:GUS plants (40 days old) were stained for GUS activity. Bar = 200 μm.

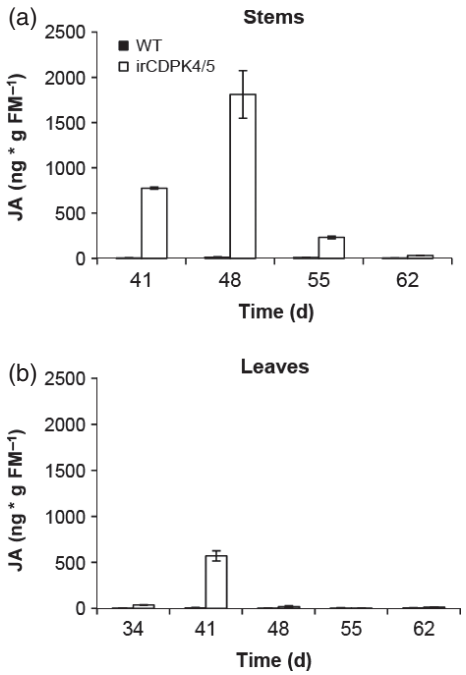


**Figure 3.** Contents of jasmonic acid (JA) in the stems and leaves of wild-type (WT) and irCDPK4/5 plants.
Wild-type and irCDPK4/5 plants were cultivated concurrently. The contents of JA (mean ± SE) were determined in their stems (a) and leaves (b) over 62 days after germination ($n$ = 5).

JA. Thus, the levels of JA were quantified using HPLC-MS/MS. The JA concentrations in WT stems ranged from 2 to 12 ng $g^{-1}$ fresh mass (FM). Strikingly, 41 and 48 days after germination, stems of irCDPK4/5 plants had 240- and 140-fold more JA than did stems of WT, respectively (Figure 3a). Greater JA levels were also found in 55- and 62-day-old plants (during senescence), although JA levels had greatly decreased at these later stages of plant development (Figure 3a). Since JA-Ile, but not JA, functions as the active jasmonate hormone (Browse, 2009), we also determined the concentrations of JA-Ile in WT and irCDPK4/5 stems and leaves (Figure S3a). High levels of JA-Ile were also detected in irCDPK4/5 stems: on days 41 and 48 after germination, irCDPK4/5 stems contained 6- and 24-fold more JA-Ile than did WT stems, respectively; more JA-Ile was also detected in irCDPK4/5 stems even in older plants (Figure S3a). Similarly, high JA and JA-Ile contents were detected in irCDPK4/5 leaf tissues; 41-day-old leaves, in particular, had strongly elevated JA contents compared with WT leaves of a similar age (94- and 13-fold greater for JA and JA-Ile, respectively; Figures 3b and S3b); however, only relatively small differences were found when plants were older than 48 days.

Enhanced JA production usually induces increased accumulation of plant secondary metabolites. Consistent with this scenario, stems of irCDPK4/5 plants rapidly turned brown after wounding, whereas WT stems showed no observable oxidative browning (Figure S4a). In line with this observation, the contents of total phenolic compounds in 41-day-old irCDPK4/5 stems were fivefold greater than those in the WT (Figure S4b). In WT stems, HPLC analysis detected only three peaks with weak signals; in contrast, samples from the stems of irCDPK4/5 showed at least 11 peaks with relatively high signal intensities (Figure S5). Among these peaks, six were identified using standards to be nicotine, caffeoylputrescine (CP), chlorogenic acid (CGA), cryptochlorogenic acid (CCA), rutin, and dicaffeoylspermidine (DCS), and another was identified to be N1-caffeoyl-N3-dihydrocaffeoylspermidine (CDCS) after fractionation and NMR analysis (Figure S5). In line with the highly increased JA levels in irCDPK4/5 stems, we found that by day 41 after germination, average nicotine content in WT stems was 436 μg $g^{-1}$ FM while it reached 2972 μg $g^{-1}$ FM in irCDPK4/5 stems; although nicotine content declined later in both types of plants, four- to five-fold greater nicotine levels were still found in irCDPK4/5 (Figure 4a). Caffeoylputrescine was undetected in WT stems, but was found in all replicates of irCDPK4/5 (Figure 4b). Chlorogenic acid exhibited increasingly greater content, and by day 62, irCDPK4/5 had approximately 86-fold more CGA (7000 and 80 μg $g^{-1}$ FM in irCDPK4/5 and WT, respectively; Figure 4c). For CCA content, we detected a maximum of 85 μg $g^{-1}$ FM in irCDPK4/5 stems on day 62 after germination, which was 12-fold higher compared with the levels in WT stems (Figure 4d). While DCS and CDCS were undetected in WT stems, large peaks of these compounds were seen in the chromatograms from irCDPK4/5 stem samples (Figure 4e, f). Notably, the concentrations of rutin were not greatly different between WT and irCDPK4/5 stems, although by day 62 the rutin content in irCDPK4/5 stems was fivefold greater (Figure 4g).

The contents of these compounds were also determined in the leaves of 48-day-old WT and irCDPK4/5 plants, when JA levels were the highest in irCDPK4/5. Although the

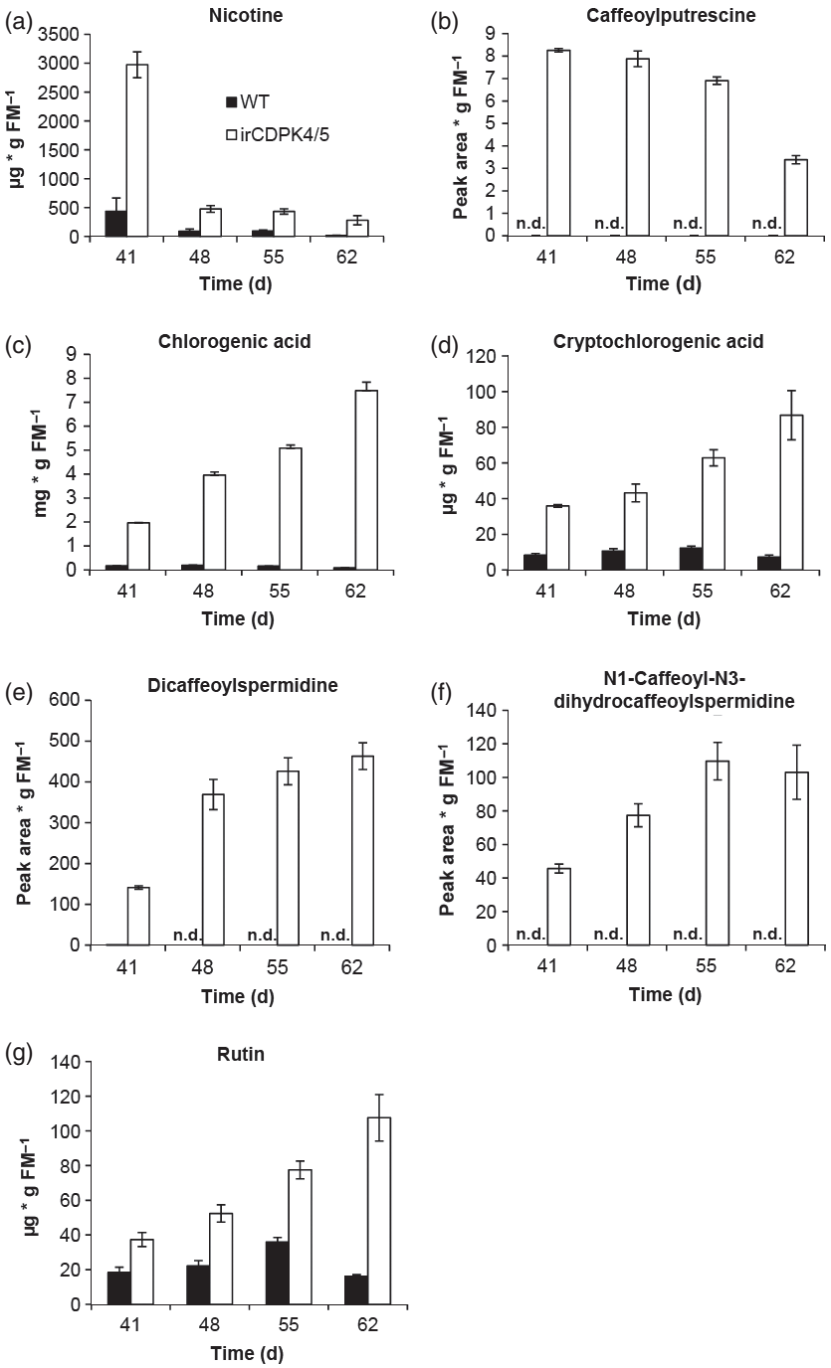


**Figure 4.** Secondary metabolite contents in the stems of wild-type (WT) and irCDPK4/5 plants. Wild-type and irCDPK4/5 plants were cultivated concurrently. The contents (mean ± SE) of nicotine (a), caffeoylputrescine (b), chlorogenic acid (c), cryptochlorogenic acid (d), dicaffeoylspermidine (e), N1-caffeoyl-N3-dihydrocaffeoylspermidine (f), and rutin (g) were measured in the stems until 62 days after germination ($n$ = 5). n.d. = not detected.

differences were not as large as those in stems, irCDPK4/5 leaves still contained more nicotine, CP, CA, CCA, DCS, and CDCS than did the leaves of WT. Conversely, there was about 40% less rutin in irCDPK4/5 leaves (Figure S6).

### Elevated JA levels in irCDPK4/5 account for the stunted stems and the accumulation of secondary metabolites

In Arabidopsis, several mutants that over-accumulate JA show stunted growth (Turner *et al.*, 2002; Bonaventure *et al.*, 2007; Hyun *et al.*, 2008). Thus, we hypothesized that the growth phenotypes of irCDPK4/5 might have resulted from the high JA levels.

To examine this hypothesis, first we applied methyl jasmonate (MeJA) to the stems of WT *N. attenuata* and recorded the stem lengths over time. As expected, applying MeJA to stems inhibited the elongation of stems (Figure 5a). In addition, a genetic approach was employed: irCDPK4/5 was crossed with the irAOC line, which is

silenced in the *ALLENE OXIDE CYCLASE* (*AOC*), an important gene encoding a JA biosynthetic enzyme (Kallenbach *et al.*, 2012). Silencing *AOC* in irCDPK4/5 plants effectively reduced JA (Figure 5b) and JA-Ile (Figure S7) levels to those in WT plants, and we found that the development of leaves and stems of irCDPK4/5 × irAOC plants was completely restored (Figure 5c). Similar developmental phenotypes of leaves and stems were also found when irCDPK4/5 was crossed with an irCOI1 line (irCDPK4/5 × irCOI1 plants; Paschold *et al.*, 2007), whose *COI1* (the receptor in JA signaling) was silenced, or with an ovJMT line (irCDPK4/5 × ovJMT plants), which ectopically expressed an Arabidopsis *JASMONIC ACID O-METHYLTRANSFERASE* (*JMT*) gene to convert JA to the inactive MeJA (Stitz *et al.*, 2011; Figure S8). In addition, the contents of secondary metabolites in irCDPK4/5 × irAOC, irCDPK4/5 × irCOI1, and irCDPK4/5 × ovJMT stems were similar to those in WT, irAOC, irCOI1, and ovJMT stems (Figures 5d–j and S9).

Thus, over-accumulated JA inhibits stem elongation and induces high levels of secondary metabolites in *N. attenuata* stems. Based on these results, we speculated that plants deficient in JA production and signaling might show longer stems. Indeed, we found that irAOC, irCOI1, and ovJMT plants exhibited about 20% longer stems than did WT (Figure S10).

**Mis-allocation of carbon sources is not likely to account for the inhibited stem growth in irCDPK4/5 plants**

It is possible that the highly enhanced accumulation of secondary metabolism diverted the limited carbon sources from being converted to growth-related compounds and thus resulted in the stunted stems of irCDPK4/5 plants.

The transcription factor MYB8 plays a critical role in regulating the biosynthesis of many major phenolic compounds and phenylpropanoid–polyamine conjugates, such as CGA, CP, and DCS (Kaur *et al.*, 2010; Onkokesung *et al.*, 2012). In order to suppress the accumulation of these compounds in irCDPK4/5 plants, we crossed irCDPK4/5 plants with the irMYB8 line to form irCDPK4/5 × irMYB8. The HPLC analysis indicated that crossing irCDPK4/5 with irMYB8 effectively abolished the accumulation of CP and CA (Figure 6a), and decreased the contents of nicotine, DCS, CDCS, and rutin 6.5-, 3.5-, 5-, and 5-fold, respectively (Figure 6a). Cryptochlorogenic acid levels did not differ between irCDPK4/5 and irCDPK4/5 × irMYB8 stem tissue (Figure 6a). Despite the decreased levels of these secondary metabolites, the morphology of irCDPK4/5 × irMYB8 plants was very similar to that of irCDPK4/5 plants (Figure 6b). From these results, we infer that it is unlikely that carbon mis-allocation resulted in the stunted stems of irCDPK4/5 plants.

**Stems of irCDPK4/5 plants are deficient in GAs**

The stunted growth of irCDPK4/5 stems and their decreased cell sizes are reminiscent of the phenotypes of GA-deficient or -insensitive plants (Sun and Gubler, 2004). Thus, bioactive $GA_3$ was applied to irCDPK4/5 plants to determine if the growth of irCDPK4/5 stems could be rescued. Twenty days after germination, when plants were still in the early rosette stage, both WT and irCDPK4/5 plants were supplied daily with 3 μM $GA_3$ for 21 days. The WT plants did not grow taller in response to the $GA_3$ treatment, but showed larger rosette and stem leaves (Figure 7). Importantly, $GA_3$ supplementation largely restored the growth of irCDPK4/5 plants, which attained 80% of the height of WT plants (Figure 7). These data suggest that deficiency of GAs is probably responsible for the stunted growth of irCDPK4/5 stems. One and 10 μM $GA_3$ were also used to examine the effect of $GA_3$ on promoting irCDPK4/5 stem growth. It was found that compared with 3 μM $GA_3$, 1 μM $GA_3$ had somewhat weaker and 10 μM $GA_3$ showed similar stem promotion effects when applied to irCDPK4/5 plants (data not shown). Although $GA_3$ supplementation largely restored the growth of irCDPK4/5 stems, it did not strongly influence the contents of secondary metabolites – $GA_3$-treated irCDPK4/5 stems still contained increased levels of CP, CA, CCA, DCS, CDCS, and rutin (Figure S11), supporting our hypothesis that carbon over-allocation to the biosynthesis of secondary metabolites only marginally accounts for the JA-inhibited stem growth in *N. attenuata*, if carbon over-allocation does affect stem growth.

Furthermore, WT, irCDPK4/5, and irCDPK4/5 × irAOC plants were grown concurrently under glasshouse conditions, and the top halves of the stems were harvested from 41-day-old plants. The levels of several GAs were determined in these samples using a gas chromatography (GC)-mass spectrometry method (Lange *et al.*, 2005). As expected, compared with those of WT plants, the GA precursors $GA_{53}$, $GA_{44}$, $GA_{19}$, and $GA_{20}$ were about 25-, 48-, 3.7-, and 10-fold lower in irCDPK4/5 plants (Table 1) and the bioactive $GA_1$ was 5.6 times lower in irCDPK4/5 plants (Table 1). After oxidation by GA2ox, $GA_1$ forms inactive $GA_8$, and $GA_8$ was also reduced in irCDPK4/5 to 30% of the WT levels, while $GA_{29}$ (its formation also requires GA2ox) showed a slight decrease (Table 1). Consistent with the fact that silencing the *AOC* gene in irCDPK4/5 abolished the suppression effect of JA on stem elongation (Figure 5c), all GAs detected in irCDPK4/5 × irAOC plants were largely at WT levels, in particular the contents of bioactive $GA_1$ being completely restored (Table 1).

The highly decreased levels of $GA_{53}$ in irCDPK4/5 stems led us to measure the content of $GA_{12}$, which is the immediate precursor of $GA_{53}$. Five replicates of WT, irCDPK4/5, and irCDPK4/5 × irAOC stems were pooled and $GA_{12}$ in these samples was detected using a GC-MS method modified from Lange *et al.* (2005; see Experimental Procedures). The average $GA_{12}$ contents in WT, irCDPK4/5, and irCDPK4/5 × irAOC stems were 0.4, 0.6, and 0.4 ng $g^{-1}$ FM, respectively (Table 1).

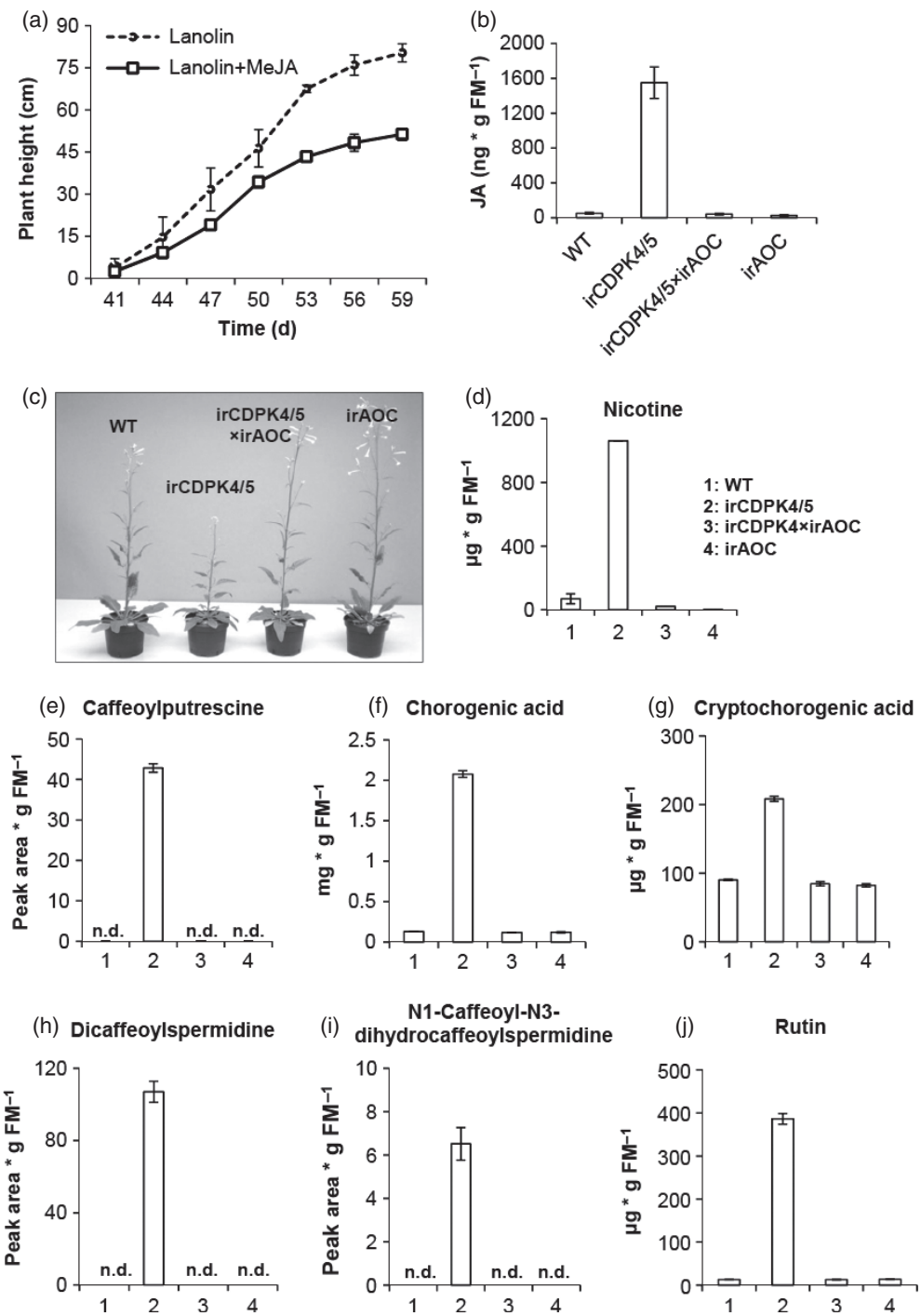


**Figure 5.** High jasmonic acid (JA) levels account for the stunted stem elongation and over-accumulation of secondary metabolites.
(a) Exogenously applying methyl jasmonate (MeJA) inhibits stem elongation. Methyl jasmonate (150 μg) in 20 μl of lanolin was applied to *Nicotiana attenuata* stems and 20 μl of pure lanolin was applied as controls ($n$ = 10). Plant heights (mean ± SE) were recorded over time.
(b) Jasmonic acid levels (mean ± SE) in the stems of 48-day-old wild-type (WT), irCDPK4/5, irCDPK4/5 × irAOC, and irAOC plants ($n$ = 5).
(c) Photograph of wild-type (WT), irCDPK4/5, irCDPK4/5 × irAOC, and irAOC plants (45 days old).
(d)–(j) Contents (mean ± SE, $N$ = 5) of nicotine, caffeoylputrescine, chlorogenic acid, cryptochlorogenic acid, dicaffeoylspermidine, N1-caffeoyl-N3-dihydrocaffeoylspermidine, and rutin in 45-day-old WT, irCDPK4/5, irCDPK4/5 × irAOC, and irAOC stems (represented by 1–4 in the $x$-axes, respectively; n.d. = not detected).

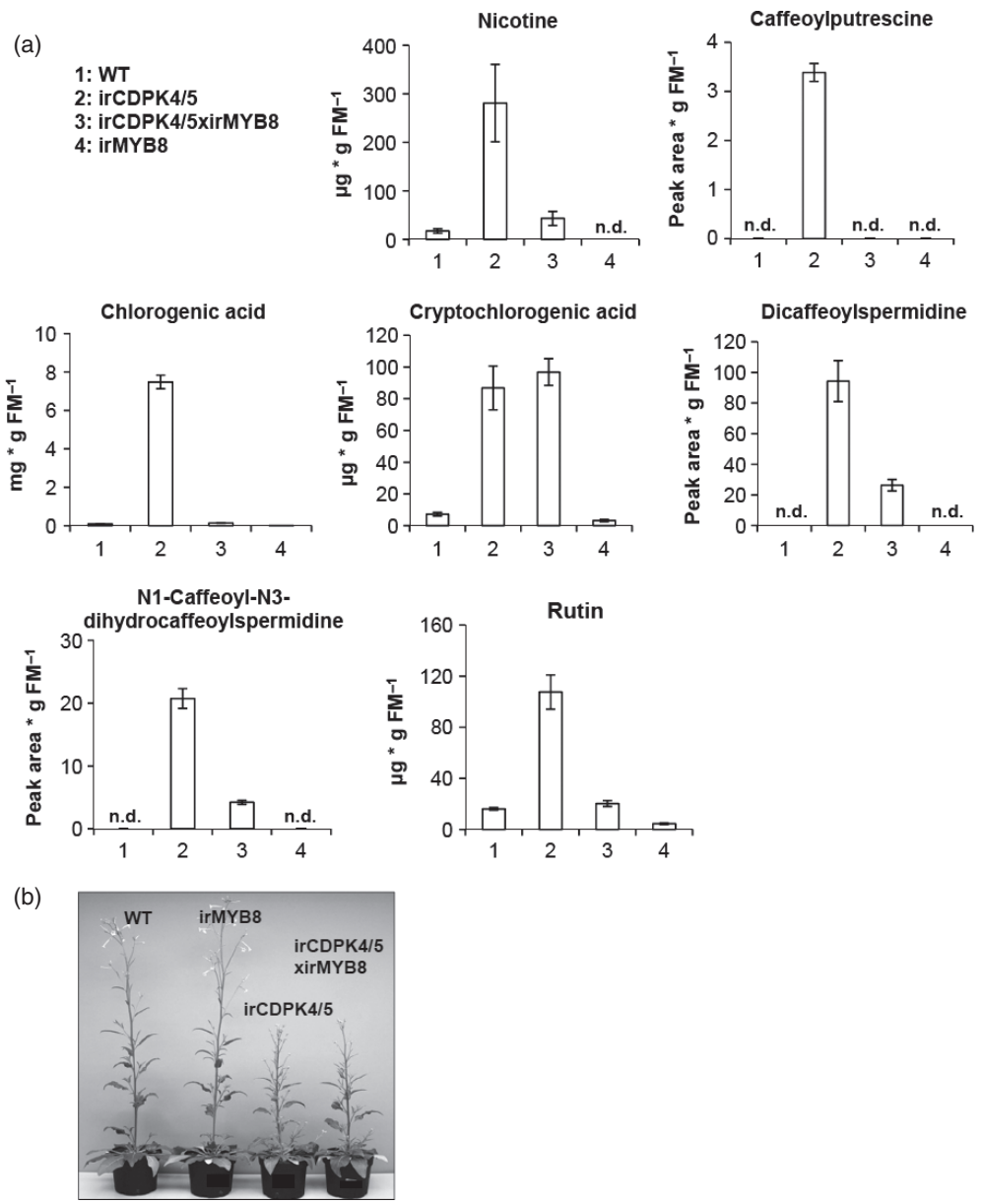


**Figure 6.** Secondary metabolites and morphologies of wild-type (WT), irCDPK4/5, irCDPK4/5 × irMYB8, and irMYB8 plants.
(a) Contents (mean ± SE) of nicotine, caffeoylputrescine, chlorogenic acid, cryptochlorogenic acid, dicaffeoylspermidine, N1-caffeoyl-N3-dihydrocaffeoylspermidine, and rutin in WT, irCDPK4/5, irCDPK4/5 × irMYB8, and irMYB8 stems (45 days old) ($n$ = 5).
(b) A photo of 45-day-old WT, irMYB8, irCDPK4/5, and irCDPK4/5 × irMYB8 plants.

### Highly elevated JA levels in irCDPK4/5 stems suppress the transcript levels of GA biosynthetic genes, including *GA20ox*

The biosynthesis of GAs has been intensively studied (Yamaguchi, 2008). It starts from geranylgeranyl diphosphate (GGDP), a common $C_{20}$ precursor for diterpenoids; *ent*-COPALYL DIPHOSPHATE SYNTHASE (CPS) and *ent*-KAURENE SYNTHASE (KS) convert GGDP to *ent*-kaurene. Sequentially, *ent*-KAURENE OXIDASE (KO) and *ent*-KAURENOIC ACID OXIDASE (KAO) catalyze the formation of $GA_{12}$ from *ent*-kaurene. Then $GA_{12}$ is further converted to various forms of active GAs ($GA_1$, $GA_3$, and $GA_4$) by GA 20-OXIDASE (GA20ox), GA 3-OXIDASE (GA3ox), and the unidentified GA 13-OXIDASE (GA13ox). Moreover, GA 2-OXIDASE (GA2ox) is involved in deactivating GAs (summarized in Figure S12).

To gain further insight into the mechanism by which the high JA levels in irCDPK4/5 might have antagonized the accumulation of GAs, first we cloned the homologs of these genes from *N. attenuata*; two GA receptor genes were also cloned: GID1b was similar to Arabidopsis GID1b,

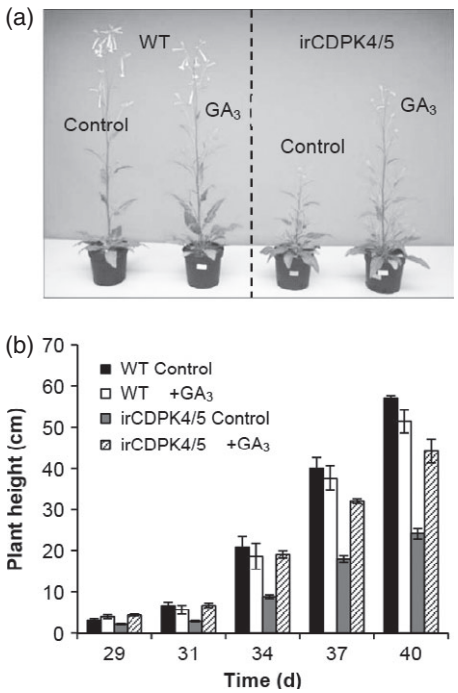


**Figure 7.** Supplementation of gibberellin $GA_3$ to irCDPK4/5 plants largely restores stem elongation.
Gibberellin $GA_3$ (3 μM) was applied daily to wild-type (WT) and irCDPK4/5 plants 20 days after germination until they were 40 days old; plants supplemented with the solvent for $GA_3$ solution served as controls.
(a) A photograph of WT and irCDPK4/5 treated with or without $GA_3$.
(b) Heights (mean ± SE) of WT and irCDPK4/5 plants after being supplemented with or without $GA_3$ ($n$ = 10).

and GID1ac showed high similarity to Arabidopsis GID1a and GID1c (for alignments see Figure S13). Quantitative RT-PCR indicated that in stems of WT and irCDPK4/5 plants, several genes involved in GA biosynthesis, deactivation, and signaling showed differences on transcript levels (Figure 8). In irCDPK4/5 stems, genes encoding enzymes in the early steps of GA biosynthesis, *CPS*, *KS*, *KO*, and *KAO*, showed no changes (for *CPS*), 20 and 30% reduction (for *KS* and *KO*, respectively), or even elevation of more than twofold (for *KAO*) in transcript levels (Figure 8a–d). GA20ox and GA3ox catalyze the last steps of GA biosynthesis. Strikingly, the transcript levels of the *GA20ox* gene in irCDPK4/5 stems were only 2% of those in WT stems (Figure 8e), but *GA3ox* transcript levels were 1.7-fold increased in irCDPK4/5 stems (Figure 8f). We also found that the transcript abundance of *GA2ox*, a gene involved in GA catabolism, was 2.7-fold greater in irCDPK4/5 stems than in WT stems (Figure 8g). While *N. attenuata GID1ac* exhibited unchanged transcript levels between irCDPK4/5 and WT stems, *GID1b* transcript abundance in irCDPK4/5 stems was elevated 11 times (Figure 8h, i).

Importantly, decreasing the levels of JA in irCDPK4/5 by crossing with irAOC plants almost fully reversed the transcript levels of all these genes (Figure 8). This confirmed that the high levels of JA in irCDPK4/5 plants accounted for the altered transcriptional levels of these GA-related genes. Furthermore, consistent with the profile of JA content in stems over time, we found that the transcript levels of *GA20ox* were the most strongly suppressed in 48-day-old irCDPK4/5 plants, when they had the highest amount of JA in stems (Figure S14). Notably, decreasing the JA levels in irAOC plants also led to enhanced transcript abundance of *GA20ox* and *GA3ox* (about 20 and 115%, respectively, compared with those in WT stems; Figure 8e, f).

Recently, Dayan *et al.* (2012) demonstrated that tobacco (*Nicotiana tabacum*) leaves are required for the accumulation of bioactive GAs in the stem and consequently for normal stem elongation. Thus, we also determined the transcript levels of these GA biosynthetic genes in leaves. Compared with those in WT leaves, no large differences

**Table 1** The contents (ng $g^{-1}$ fresh mass) of endogenous gibberellins (GAs) in the upper parts of 41-day-old wild-type (WT), irCDPK4/5, and irCDPK4/5 × irAOC stems

| | WT | | | irCDPK4/5 | | | irCDPK4/5 × irAOC | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| $GA_{53}$ | 1.93 | 0.93 | 0.86 | n.d. | 0.03 | 0.11 | 0.36 | 0.25 | 0.47 |
| $GA_{44}$ | 3.40 | 3.16 | 2.19 | 0.01 | 0.09 | 0.08 | >10 | 2.64 | 2.18 |
| $GA_{19}$ | 7.33 | 6.48 | 5.49 | 1.42 | 2.56 | 1.21 | 5.92 | n.d. | 4.71 |
| $GA_{20}$ | 2.68 | 3.12 | 2.90 | 0.85 | 0.88 | 0.90 | 2.18 | 1.81 | 1.68 |
| $GA_1$ | 0.70 | 0.35 | 0.23 | 0.06 | 0.07 | 0.10 | 0.51 | 0.19 | 0.33 |
| $GA_8$ | >10 | 3.61 | 3.94 | 1.19 | 1.56 | 1.04 | 7.70 | 3.71 | >10 |
| $GA_{29}$ | >10 | 9.41 | 7.44 | 6.12 | 7.05 | 3.57 | >10 | 9.39 | 9.77 |
| $GA_{12}$[a] | | 0.4 | | | 0.6 | | | 0.4 | |

n.d., not determined.
1–3 represent three biological replicates.
[a]For $GA_{12}$ measurement, five biological replicates from each type of plants were individually harvested and ground and the samples were subsequently mixed to obtain an average value of $GA_{12}$ content.

 

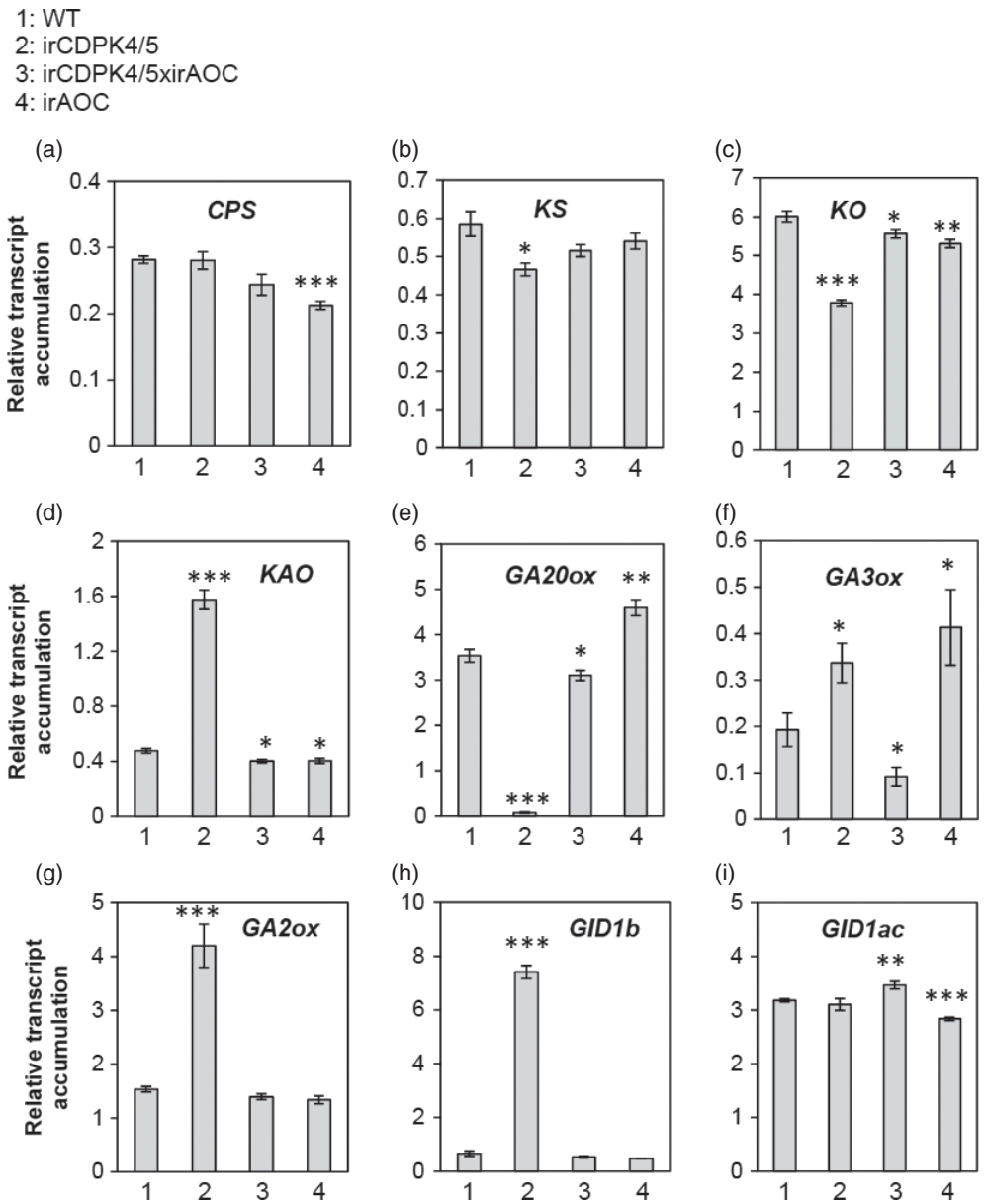


**Figure 8.** Transcript levels of genes in the stems of wild-type (WT), irCDPK4/5, irCDPK4/5 × irAOC, and irAOC plants.
Stems from 41-days-old plants were harvested and quantitative RT-PCR was done to quantify transcript levels (mean ± SE) of (a) *CPS*, (b) *KS*, (c) *KO*, (d) *KAO*, (e) *GA20ox*, (f) *GA3ox*, (g) *GA2ox*, (h) *GID1b*, and (i) *GID1ac* genes. Asterisks indicate significant differences between WT and irCDPK4/5, irCDPK4/5 × irAOC, or irAOC plants (*t*-test; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $n = 5$).

were found, except that *GA20ox*, *GA3ox*, and *GA2ox* respectively showed 35, 60, and 47% decreased transcript levels in irCDPK4/5 leaves (Figure S15). Thus, it is likely that irCDPK4/5 leaves have only minor decreases in GAs, and these are unlikely to account for the highly reduced GAs in stems.

Given the large differences between the transcript levels of *GA20ox* in WT and irCDPK4/5 stems and the critical role of *GA20ox* in GA biosynthesis, we inferred that JA antagonizes the biosynthesis of GAs mainly by inhibiting the transcript accumulation of GA biosynthetic genes, including *GA20ox*.

### *GA20ox* plays an important role in *N. attenuata* stem growth

To test the hypothesis that the decreased transcript levels of *GA20ox* were responsible for the stunted stems of irCDPK4/5 plants, a virus-induced gene silencing (VIGS) approach was used to knock down *GA20ox* expression. A partial sequence of *GA20ox* was cloned into the vector

pTV00 to form pTV-GA20ox, and *Agrobacterium tumefaciens* harboring pTV-GA20ox was subsequently inoculated into *N. attenuata* to silence *GA20ox*; plants inoculated with *A. tumefaciens* carrying pTV00 (empty vector) were used for comparisons (these plants were named VIGS-GA20ox and EV plants, respectively). Quantitative RT-PCR analysis indicated that the transcript abundance of *GA20ox* in the stems of VIGS-GA20ox plants was around 18% of that in the stems of EV plants (Figure 9a). As expected, compared with those of EV plants, VIGS-GA20ox exhibited reduced stem lengths (about 40%; Figure 9b), confirming the important role of GA20ox in stem elongation.

## DISCUSSION

Plant development, growth, and stress responses are largely orchestrated by various hormone pathways and these pathways often have synergistic or antagonistic cross-talk to fine-tune plant physiology (Grant and Jones, 2009; Depuydt and Hardtke, 2011). Increased levels of JA are detected in plants challenged with certain biotic and abiotic stresses, such as wounding, herbivore feeding, and infections from necrotic fungi (Wasternack, 2007; Wu and Baldwin, 2010), and the increased JA levels are usually associated with enhanced defense but also decreased growth (Baldwin, 1998; Zhang and Turner, 2008). However,

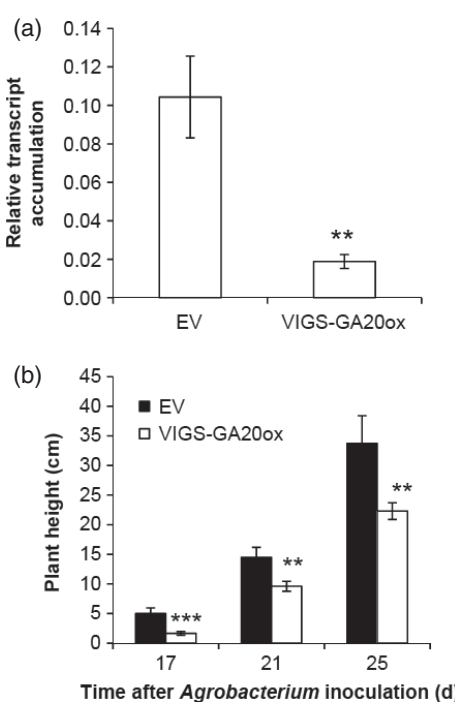


**Figure 9.** Silencing *GA20ox* in *Nicotiana attenuata* results in stunted stem elongation. A virus-induced gene silencing (VIGS) system was used to create EV (empty vector) and VIGS-20ox plants.
(a) Transcript levels of *GA20ox* in EV and VIGS-GA20ox plants ($n = 5$).
(b) Heights (mean ± SE) of EV and VIGS-GA20ox plants ($n = 10$). Asterisks indicate significant differences between WT and irCDPK4/5 plants (*t*-test; $^{**}P < 0.01$).

how an increased JA content suppresses plant growth was unclear. Using irCDPK4/5 plants, which were silenced in the JA suppressors, *CDPK4* and *CDPK5*, and thus had dramatically increased levels of JA, we show that JA signaling represses the biosynthesis of GAs by inhibiting the transcription of several GA biosynthetic genes, including *GA20ox*, which encodes a key enzyme catalyzing the formation of bioactive GAs. Furthermore, we provide evidence that suppressed GA production is likely largely responsible for the decreased plant growth, but not the diverted resources for the biosynthesis of secondary metabolites.

### CDPK4 and CDPK5 are critical repressors of JA biosynthesis in stems and leaves

Although almost all enzymes in JA biosynthesis have been cloned (Wasternack, 2007), little is still known about how JA biosynthesis is regulated. In *N. attenuata*, although the underlying mechanisms remain unclear, MAPKs (Wu *et al.*, 2007), receptors BAK1 and COI1 (Paschold *et al.*, 2008; Yang *et al.*, 2011), nitric oxide (Wünsche *et al.*, 2011a,b), and the co-chaperone SGT1 (Meldau *et al.*, 2011) all influence JA levels. In Arabidopsis, a mutant carrying a missense mutation in the *TWO PORE CHANNEL 1* (*TPC1*) gene, which encodes a $Ca^{2+}$-permanent non-selective cation channel, shows highly accumulated JA, implicating $Ca^{2+}$ in JA biosynthesis (Bonaventure *et al.*, 2007). In tomato the *AOC* gene is expressed in vascular bundles (Stenzel *et al.*, 2003), and consistent with the function of CDPK4/5 in stems and in JA biosynthesis, we detected strong activity of CDPK4/5 promoters in the vascular tissues in stems. We speculate that in *N. attenuata* stems and leaves, $Ca^{2+}$ might be involved in JA regulation, since CDPKs are activated by $Ca^{2+}$.

Compared with WT *N. attenuata*, simultaneously silencing *CDPK4/5* increases wounding- and *M. sexta* herbivory-induced JA by about 5.25- and 2.4-fold respectively (Yang *et al.*, 2012a). Here we also show that CDPK4/5 are also involved in suppressing the accumulation of JA in leaves and stems during development (Figure 3). Compared with their roles in controlling wounding- and herbivory-induced JA, their function in suppressing the biosynthesis of JA in stems and leaves seems likely to be more important, given that the highest levels of JA in irCDPK4/5 stems were more than 200 times greater than those in WT stems. Furthermore, the JA content in irCDPK4/5 stems was associated with plant growth stages – JA content was dramatically greater during stem elongation (usually between 33 and 50 days after germination) than in older plants when stem growth had almost stopped. A similar dependency was also observed for leaves: leaves of 41-day-old irCDPK4/5 had very high JA levels, while the JA content in younger or older irCDPK4/5 leaves was not so strongly different from that in WT leaves. Moreover, CDPK4/5 seem to play a

 

more important role in stems than in leaves, considering that the high levels of JA appeared more transiently in leaves. It is likely that during the rapid stem and leaf elongation process, CDPK4/5 are important suppressors that mediate JA homeostasis and therefore relieve the antagonistic effect of JA on GA biosynthesis to supply a normal amount of GAs for stem elongation.

The importance of CDPK4/5 in maintaining JA homeostasis is also demonstrated by the over-accumulation of secondary metabolites in irCDPK4/5 stems. In WT plants, stems contain very low amounts of detectable secondary metabolites, even after leaves have been wounded or attacked by herbivores (C. Hettenhausen, M. Heinrich, I. T. Baldwin and J. Wu, unpublished data). However, several compounds, which are normally not detectable in stems, accumulated to a high level in irCDPK4/5 stems, and this was due to the over-production of JA, since silencing *AOC* in irCDPK4/5 completely reversed the contents of these compounds to those found in WT or irAOC plants. Moreover, these data also suggest that under certain conditions, stems are able to synthesize various secondary metabolites and these could even be produced in high quantity (Figures 4 and S5). The possibility that these compounds were produced in leaves and transported to stems cannot be ruled out.

**The biosynthesis of GAs is antagonized by JA**

At least three Arabidopsis mutants, *cev1*, *fou2*, and *dgl-D*, produce an excessive amount of JA, and these plants all have stunted growth (Ellis *et al.*, 2002; Bonaventure *et al.*, 2007; Hyun *et al.*, 2008). Crossing *dgl-D* with *opr3* or *coi1* to abolish its JA biosynthesis or signaling effectively restored its normal vegetative growth (Hyun *et al.*, 2008). Similarly, silencing *AOC* or *COI1* or ectopically overexpressing the *JMT* gene in irCDPK4/5 also rescued stem elongation. Thus, high levels of JA inhibit plant growth and the defects in the growth of irCDPK4/5 resulted only from the over-accumulated JA but not from other pathways that CDPK4 and/or CDPK5 may regulate.

One scenario of how JA inhibits plant growth is that JA may divert the allocation of carbon and nitrogen resources to defense/secondary metabolites and thereby reduce plant growth. When irCDPK4/5 plants were crossed with irMYB8 plants (deficient in an important transcription factor for the biosynthesis of phenolic compounds and phenylpropanoid –polyamine conjugates), we could effectively decrease the contents of most of the detectable secondary metabolites, but this did not restore the normal growth of irCDPK4/5 plants. Therefore, it is very likely that carbon mis-allocation only accounts for minor aspects of the decreased stem elongation of irCDPK4/5 and the highly decreased GAs were mainly responsible for the stunted stem growth.

The bioactive $GA_1$ is formed from $GA_{12}$, $GA_{53}$, $GA_{44}$, $GA_{19}$, and $GA_{20}$, consecutively (Figure S12). Quantification of $GA_{12}$, one of the early precursors in the biosynthesis pathway, revealed that $GA_{12}$ was only slightly elevated in irCDPK4/5 stems compared with WT stems, suggesting that high JA levels do not suppress the activity of CPS, KS, KO, and KAO. In contrast, the $GA_{53}$ contents were greatly decreased (25-fold) in irCDPK4/5 stems (Table 1); given that the formation of $GA_{53}$ requires the yet-to-be-identified GA13ox, it is very likely that high JA levels greatly reduce the transcription of *GA13ox* and suppress its activity. Furthermore, the enzyme GA20ox converts $GA_{53}$ sequentially to form $GA_{44}$, $GA_{19}$, and $GA_{20}$. Among all the GA biosynthetic genes that we examined, *GA20ox* transcript levels were only 2% of those in WT stems, and silencing JA biosynthesis or signaling recovered *GA20ox* transcript levels. Thus the over-accumulation of JA also strongly suppresses *GA20ox* transcript accumulation, and it is conceivable that GA20ox enzyme activity was also greatly reduced in irCDPK4/5 stems. We propose that in addition to GA13ox, the reduced activity of GA20ox probably also contributes to the impaired production of $GA_{44}$, $GA_{19}$, and $GA_{20}$. The reduced $GA_{20}$ content is likely to be the reason for the low $GA_1$ levels in irCDPK4/5 stems. Given that, like $GA_1$, bioactive $GA_3$ is also derived from $GA_{20}$, and the production of bioactive $GA_4$ also requires GA20ox, it is likely that the concentrations of these two GAs are also reduced in irCDPK4/5 stems, although we were not able to determine their concentrations due to technical difficulties in the measurements. Whether *GA20ox* belongs to a multigene family in *N. attenuata* cannot be ruled out so far. Using a VIGS system, we further confirmed that *GA20ox* is important for promoting stem growth in *N. attenuata*. Changes of transcript levels in other genes may also have contributed to the decreased GA content in irCDPK4/5 plants, for example the increased *GA2ox* gene (encoding a GA-deactivating enzyme) transcripts.

Under optimal growth conditions, transgenic *N. attenuata* plants deficient in JA or JA signaling are taller than WT plants (Figure S10). Compared with WT, JA-deficient irAOC plants had more than twice as many transcripts of *GA3ox* (Figure 8F), and this may lead to slightly increased bioactive GAs and thereby enhance plant growth. Supporting this hypothesis, silencing *COI1* in rice increases the levels of $GA_4$ by 3.8-fold in the elongating uppermost internode (Yang *et al.*, 2012b). Notably, in irCDPK4/5 plants, the elevated JA content in stems suppressed at least $GA_1$, whereas in rice silencing *COI1* increased the levels of $GA_4$ but not $GA_1$ (Yang *et al.*, 2012b). It would be interesting to determine whether JA signaling affects different branches of GA biosynthesis in monocots and dicots.

It is still unclear how JA signaling suppresses the transcript levels of *GA20ox*. Besseau *et al.* (2007) found that in Arabidopsis over-accumulation of flavonoids leads to inhibited auxin transport and reduced plant size. Recently, it was found that in roots of Arabidopsis seedlings MeJA

inhibits endocytosis and the accumulation of PIN-FORMED 2 (PIN2), which is an auxin efflux carrier (Sun *et al.*, 2011). Furthermore, an important transcription factor regulated by JA, MYC2, binds to the promoters of *PLETHORA1* (*PLT1*) and *PLT2*, thereby suppressing their expression and resulting in decreased auxin-induced regulation of stem cell niche maintenance in roots of Arabidopsis seedlings (Chen *et al.*, 2011). Further study is needed on whether a small molecule or a protein, which is induced by JA, acts as a suppressor of the transcription of *GA13ox* and *GA20ox* or an enhancer of these transcripts' turn-over.

In Arabidopsis and rice, transcript accumulations of several GA biosynthesis and deactivation genes are regulated by the levels of bioactive GAs and GA signaling, enabling plants to modulate GA homeostasis (reviewed in Yamaguchi, 2008). The transcript abundance of the receptor for GAs, *GID*, is also feedback-regulated by GAs (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006). In irCDPK4/5 stems, we found increased transcript levels of *KAO*, *GA2ox*, *GA3ox*, and *GID1b*. Therefore, we propose that many GA biosynthesis and signaling genes might be controlled transcriptionally, at least by both JA and GA signaling.

Recently, Yang *et al.* (2012b) demonstrated that in rice, JA antagonizes GA signaling: *COI1*-silenced rice plants grow taller than do WT plants, have increased sensitivity to GA, and exogenous application of JA also suppressed GA-induced elongation of the second leaf sheath. However, the responsiveness of irCDPK4/5 to exogenously applied $GA_3$ suggests that in *N. attenuata* JA may have no or only little inhibitory effect on GA signaling, and in contrast, probably due to the feedback regulatory pathway between GAs and the receptor GID1, the low GA contents in irCDPK4/5 induced more than a 10-fold increase in *GID1b* transcripts. Thus, irCDPK4/5 may not have had suppressed GA signaling and might even have had greater sensitivity to GAs than did WT plants, and this might be the reason why irCDPK4/5 responded more strongly to the supplementation of $GA_3$ than did WT plants (Figure 7). Unlike *COI1*-silenced rice, the Arabidopsis mutant *coi1* showed a height not different from WT (Zhang and Turner, 2008; Browse, 2009; Yang *et al.*, 2012b), suggesting that in rice and Arabidopsis JA signaling may have somewhat different effects on GA signaling in stem elongation. Therefore, it is possible that plants may have evolved different mechanisms to allow JA to suppress GA-modulated growth, either by decreasing the accumulation of bioactive GAs (this study) or by repressing GA signaling (Yang *et al.*, 2012b).

In rice and Arabidopsis, Yang *et al.* (2012b) found that JA suppresses GA responses through antagonizing GA-mediated degradation of DELLAs. Here we provide evidence that JA signaling also antagonizes GA accumulation by at least suppressing the transcript accumulation of *GA13ox* and *GA20ox*. We proposed that plant growth inhibition induced by wounding, herbivore feeding, or necrotic fungal infection is at least partly due to the reduced GA levels caused by increased JA after these challenges. Jasmonic acid promotes the biosynthesis of auxin (Dombrecht *et al.*, 2007; Sun *et al.*, 2011) and affects auxin distribution and signaling (Chen *et al.*, 2011). Thus, JA may also cross-talk with other hormones that are involved in growth regulation, such as BRs and CKs, and affect plant growth.

## EXPERIMENTAL PROCEDURES

### Plant materials

The creation of irCDPK4/5, CDPK4Pro:GUS, and CDPK5Pro:GUS plants is described elsewhere (Yang *et al.*, 2012a). *Nicotiana attenuata* WT seeds were originally collected in Utah (USA) and had been inbred for 31 generations in the glasshouse. Seed germination and plant cultivation followed Krügel *et al.* (2002). Crossing irMYB8, irAOC, irCOI1, and ovJMT with irCDPK4/5 plants was done by removing anthers before pollen maturation and hand-pollinating the stigmas with irCDPK4/5 pollen.

### Microscopy analyses

Histochemical GUS assays were done following Jefferson *et al.* (1987). Stem cross sections (20 μm thick) from CDPK4Pro:GUS and CDPK5Pro:GUS plants were dissected using a vibratome (HM 650V; MICROM International GmbH, http://www.microm-online.com/) and cross sections were immediately fixed in ice-cold 90% acetone for 1 h; thereafter sections were washed with water and were immersed in the enzymatic reaction mixture (1 mg $ml^{-1}$ of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 2 mM ferricyanide, and 0.5 mM of ferrocyanide in 100 mM phosphate buffer, pH 7.4). The reaction was performed at 37°C in the dark for 5–30 min, and then the samples were cleared with pure ethanol. Pictures were taken on a microscope (Leica DM6000 B; Leica Microsystems GmbH, http://www.leica-microsystems.com/).

For observation of pith cells, cross sections (20 μm thickness) of WT and irCDPK4/5 stems were prepared on a vibratome (MICROM HM 650V). Toluidine blue was used to stain the cells (Obrien *et al.*, 1964) and microscope images were taken on a microscope (Leica DM6000 B). Cell size was calculated using ImageJ (http://rsbweb.nih.gov/ij/).

### Plant treatment

For complementation assays, plants were sprayed with 3 μM $GA_3$ once a day for 21 days after plants were 20 days old and plants sprayed with 0.01% ethanol were used for comparisons. For mimicking high JA levels, *N. attenuata* stems were treated every third day with 20 μl of lanolin paste containing 150 μg of MeJA; plants similarly treated with pure lanolin were used for controls.

### RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from leaves was extracted from ground samples using TRIzol reagent (Invitrogen, http://www.invitrogen.com/) following the manufacturer's instructions. For RNA extraction from stem tissues we used a lithium chloride method described in (Kistner and Matamoros, 2005). For quantitative RT-PCR analysis, five replicated biological samples were used. Total RNA samples (0.5 μg) were reverse-transcribed using oligo$(dT)_{18}$ and Superscript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed on an a Mx3005P system (Stratagene, http://www.strat-

 

agene.com) and a quantitative PCR Core Kit for SYBR® Green I (Eurogentec, http://www.eurogentec.com). Transcript levels were normalized using an *N. attenuata ELONGATION FACTOR 1A* (*EF1a*) gene. All primer sequences used for quantitative RT-PCR are listed in Table S1. GenBank accession numbers for all sequences can be found in Table S2.

### Phytohormone analysis

For JA analysis, frozen plant materials (100 mg) were homogenized in 2-ml microcentrifuge tubes containing two metal balls and 1 ml of ethyl acetate spiked with 200 ng of $D_2$-JA. Homogenization was done twice with 200 strokes $min^{-1}$ for 1 min using a Geno/Grinder 2000 (SPEX CertiPrep, http://www.spexcsp.com/). Samples were centrifuged at 13 000 ***g*** for 20 min at 4°C. Supernatants were dried using a vacuum concentrator (Eppendorf AG, http://www.eppendorf.com/). The residues were resuspended in 500 μl of 70% methanol by vortexing for 5 min, and centrifuged 10 min at 4°C (13 000 ***g***). Supernatants were transferred to crimp vials and sample measurements were carried out as described in Wu *et al.* (2007).

Gibberellins were measured essentially according to Lange *et al.* (2005). From 41-day-old plants, the upper halves of the stems (including shoot meristem) from five plants were harvested in liquid nitrogen and ground and pooled as one replicate; three of these replicates from each type of plant were prepared for gibberellin analysis. For $GA_{12}$ measurement, five biological replicates from each type of plant were individually harvested and ground in liquid nitrogen and were subsequently mixed to obtain an average value of the $GA_{12}$ content. Moreover, the extraction protocol was slightly modified as follows: methanol was replaced by acetone, and the solvent partition procedure at pH 8.0 was omitted; the $GA_{12}$ data were obtained from the same 41-day-old plants.

### Analysis of secondary metabolites

About 200 mg of plant tissue, which was briefly ground in liquid nitrogen, was homogenized in 2-ml microcentrifuge tubes containing 1 ml of 80% methanol with 0.05% acetic acid using a Geno/Grinder 2000 (200 strokes $min^{-1}$ for 1 min). Samples were further centrifuged 20 min at 4°C for 15 min, and the supernatants were measured using a HPLC method described in Keinanen *et al.* (2001).

### Purification and identification of N1-caffeoyl-N3-dihydrocaffeoylspermidine

Purification and NMD structural elucidation of N1-caffeoyl-N3-dihydrocaffeoylspermidine is described in Methods S1.

### Molecular cloning and virus-induced gene silencing

Primers used for cloning GA biosynthetic genes and receptors are listed in Table S3.

A partial *GA20ox* (GenBank accession number JQ413251) sequence was amplified using a plasmid containing *GA20ox* gene as the templates and gene-specific primers (Table S4). The PCR products were digested with appropriate restriction endonucleases and were further ligated into pTV00 to obtain pTV-GA20ox. *Agrobacterium tumefaciens* carrying this construct was inoculated into *N. attenuata* to obtain gene-silenced plants following a procedure optimized for *N. attenuata* (Saedler and Baldwin, 2004). Plants inoculated with *A. tumefaciens* carrying pTV00 (empty vector) were used for comparisons (the EV plants). Plants silenced in *PHYTOENE DESATURASE* (*PDS*) were used to monitor the degree of VIGS, since these plants show a photobleaching phenotype (Saedler and Baldwin, 2004). About 14 days after inoculation, when the leaves of *PDS*-silenced plants were completely white, growth measurements were performed.

## ACKNOWLEDGEMENTS

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** irCDPK4/5 plants have stunted stems and reduced rosette leaf sizes.

**Figure S2.** Stems of irCDPK4/5 plants have decreased pith cell sizes.

**Figure S3.** Content of jasmonic acid-isoleucine conjugate (JA-Ile) in the stems and leaves of wild-type and irCDPK4/5 plants.

**Figure S4.** irCDPK4/5 stems have high levels of phenolic compounds.

**Figure S5.** Overlaid HPLC chromatograms of the secondary metabolites in wild-type, irCDPK4/5, irCDPK4/5 × irCOI1, and irCOI1 stem tissues.

**Figure S6.** Secondary metabolites in the leaves of wild-type and irCDPK4/5 plants.

**Figure S7.** The content of jasmonic acid-isoleucine conjugate (JA-Ile) in the stems of wild-type, irCDPK4/5, irCDPK4/5 × irAOC, and irAOC plants.

**Figure S8.** The growth phenotypes of wild-type, irCDPK4/5, irCDPK4/5 × irCOI1, irCDPK4/5 × ovJMT, irCOI1, and ovJMT plants.

**Figure S9.** Contents of secondary metabolites in the stems of wild-type, irCDPK4/5, irCOI1, ovJMT, irCDPK4/5 × irCOI1, and irCDPK4/5 × ovJMT plants.

**Figure S10.** Plant heights are correlated with jasmonic acid levels.

**Figure S11.** Secondary metabolites in wild-type and irCDPK4/5 stems after supplementation of gibberellin $GA_3$.

**Figure S12.** A simplified pathway of gibberellin biosynthesis.

**Figure S13.** Alignments of protein sequences.

**Figure S14.** *GA20ox* transcript levels in wild-type and irCDPK4/5 *Nicotiana attenuata* stem.

**Figure S15.** Transcript levels of genes in the leaves of wild-type, irCDPK4/5, irCDPK4/5 × irAOC, and irAOC plants.

**Table S1** Sequences of primers used for quantitative RT-PCR.

**Table S2** GenBank accession numbers.

**Table S3** Primers used for cloning gibberellin biosynthesis and receptor genes.

**Table S4** Primer used to clone *GA20ox* into pTV00 to obtain the virus-induced gene silencing construct.

**Methods S1.** Isolation and identification of N1-caffeoyl-N3-dihydrocaffeoylspermidine.

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