Jasmonate signaling involves the abscisic acid receptor PYL4 to regulate metabolic reprogramming in *Arabidopsis* and tobacco

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The phytohormones jasmonates (JAs) constitute an important class of elicitors for many plant secondary metabolic pathways. However, JAs do not act independently but operate in complex networks with crosstalk to several other phytohormonal signaling pathways. Here, crosstalk was detected between the JA and abscisic acid (ABA) signaling pathways in the regulation of tobacco (*Nicotiana tabacum*) alkaloid biosynthesis. A tobacco gene from the PYR/PYL/RCAR family, *NtPYL4*, the expression of which is regulated by JAs, was found to encode a functional ABA receptor. NtPYL4 inhibited the type-2C protein phosphatases known to be key negative regulators of ABA signaling in an ABA-dependent manner. Overexpression of *NtPYL4* in tobacco hairy roots caused a reprogramming of the cellular metabolism that resulted in a decreased alkaloid accumulation and conferred ABA sensitivity to the production of alkaloids. In contrast, the alkaloid biosynthetic pathway was not responsive to ABA in control tobacco roots. Functional analysis of the *Arabidopsis* (*Arabidopsis thaliana*) homologs of *NtPYL4*, *PYL4* and *PYL5*, indicated that also in *Arabidopsis* altered *PYL* expression affected the JA response, both in terms of biomass and anthocyanin production. These findings define a connection between a component of the core ABA signaling pathway and the JA responses and contribute to the understanding of the role of JAs in balancing tradeoffs between growth and defense.

nicotine | phenylpropanoid | primary metabolism | secondary metabolism | stress response

Plant hormones operate in a complex framework of interacting responses rather than through isolated linear pathways. This hormonal "crosstalk" network can be modulated by a multitude of signals from developmental or environmental origins. The integration of these signals defines the amplitude and the specificity of the responses in different cellular contexts (1–6).

Plant responses to several (a)biotic stresses involve the jasmonates (JAs) as one of the key hormonal components but also implicate the action of salicylic acid, ethylene, abscisic acid (ABA), auxin, and gibberellic acid (GA), the signaling pathways of which interact with that of the JAs to ensure a timely, spatially, and fitness-cost correct response (2, 3, 6, 7). JAs are particularly renowned for their prominent and universal role in the regulation of plant metabolism, which is typically manifested as the elicitation of secondary metabolite biosynthesis when plants face particular environmental stresses (6, 8). For instance, in common tobacco (*Nicotiana tabacum*), JAs, of which the synthesis is induced *in planta* by herbivore attack (9), can stimulate the production of pyridine alkaloids (10, 11).

When JAs, such as jasmonic acid (JA) and methyl jasmonate (MeJA), are applied to plants or produced endogenously from linolenic acid, they are converted into the biologically active form (+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile), which is subsequently bound to its receptor, the SCF^{COI1} complex that contains the CORONATINE INSENSITIVE1 (COI1) F-box protein (12, 13). This hormone–receptor interaction causes degradation of the JAZ repressor proteins by the 26S proteasome, which in turn releases activator proteins such as MYC2, MYC3, and MYC4 in *Arabidopsis* to activate distinct JA response genes (14–16). In the absence of bioactive JAs, the JAZ repressor proteins bind to the MYC factors and recruit the NINJA-TOPLESS corepressor complex to inhibit MYC activity (17).

In *Arabidopsis*, the MYC, JAZ, and COI1 proteins compose the core JA signaling machinery and have been shown to serve as a point of crosstalk with other signaling pathways. For instance, the DELLA proteins, the central repressors of the GA pathway, can manipulate MYC2 activity through competitive binding to the JAZ proteins, thereby modulating the JA signaling (18). The central regulators of the auxin signaling pathway, the auxin/indole-3-acetic acid (AUX/IAA) and auxin response transcription factors, have been shown to control the expression of the *JAZ1* gene, enabling molecular interplay between auxin and JA signaling (19). Furthermore, the auxin, the JA, and the ABA pathways all can recruit the TOPLESS corepressor proteins through the interaction with specific adaptor proteins: the AUX/IAA, NINJA, and the AFP proteins, respectively (17). Crosstalk between the ABA and JA signaling pathways can also occur through MYC2 because this transcription factor was originally described as an activator of ABA signaling (20) before being found to play a critical role in JA signaling (21). Furthermore, besides the recruitment of MYC2 and TOPLESS, that of other signaling proteins, such as the MAP kinase MPK6 (22, 23) and the AUXIN-RESISTANT 1, a subunit of the RUB1-activating enzyme that regulates the protein degradation activity of Skp1–Cullin–F-box complexes (24), might possibly enable crosstalk between the ABA and JA signaling pathways.

Similarly to the JAs, ABA plays an important role in several stress responses (2, 4, 25–27). ABA affects the biosynthesis of several osmocompatible solutes and secondary metabolites, such

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as that of terpenoid indole alkaloids in *Catharanthus roseus* (28) and anthocyanins in *Arabidopsis* (*Arabidopsis thaliana*) (29), both pathways that are also controlled by JAs in these plants. Antagonistic as well as synergistic interactions can occur between ABA and JA: in *Arabidopsis*, for instance, endogenous ABA can have distinct regulatory effects on the JA-responsive insect and pathogen response (7, 25, 30). Validated by the recent identification of the PYR/PYL/RCAR proteins, a model for ABA action has been established (27, 31–33). The PYR/PYL/RCAR proteins function as direct ABA receptors that recruit group A PP2C proteins after ABA binding and thereby inhibit their function. In the absence of ABA, these PP2C phosphatases are negative regulators of ABA signaling, because they inhibit the SnRK2 kinases that, in turn, act as the global positive regulators of ABA-induced gene expression by phosphorylating downstream transcription factors and/or other regulatory proteins (27).

Here we identified NtPYL4, a member of the PYR/PYL/RCAR protein family, from tobacco and show that it acts as an ABA receptor in a signaling network conserved between *Arabidopsis* and tobacco. Our results define a connection between the core ABA signaling pathway and the JA responses to monitor elicitor-induced reprogramming of plant metabolism and growth.

Results

NtPYL4 Is a Functional ABA Receptor and an Ortholog of the *Arabidopsis* PYL4 Protein. Previously, a gene collection had been established, based on a transcriptome analysis of MeJA-elicited tobacco cells to identify proteins involved in alkaloid biosynthesis (10). Several functional screens revealed an AP2/ERF transcriptional activator of nicotine biosynthesis (34), a nicotine transporter (35), and a GH3-like enzyme that reduces bioactive auxin levels, thereby impeding the negative crosstalk of that pathway and stimulating nicotine accumulation in tobacco roots (36). Here we focus on another gene from that collection, the initially labeled *C17* gene (European Molecular Biology Laboratory accession no. AJ966358) (10, 34) that was renamed *NtPYL4* because a BLAST search combined with a ClustalW sequence alignment revealed that it encoded a protein of 213 amino acids that belonged to the PYR/PYL/RCAR family of *Arabidopsis* ABA receptors and has the highest similarity to the PYL4/PYL5/PYL6 branch, particularly with PYL4 (Fig. 1*A* and Fig. S1). The correlation of the ClustalW multiple sequence alignment and the secondary structure of *Arabidopsis* PYR1, obtained from its crystal structure (37) (http://espript.ibcp.fr/ESPript/ESPript/), predicted that the secondary structure of NtPYL4 was similar to the characteristic Bet v 1/START fold of the intracellular PYR/PYL/RCAR ABA receptors (Fig. 1*A* and Fig. S1) (31–33). Although the PYR/PYL/RCAR receptors, as well as the clade-A PP2Cs that they inhibit, are conserved in different plant species, direct proof for the function of these ABA receptors in crop plants is still lacking. To provide functional evidence for the role of NtPYL4 as an ABA receptor, we assayed its interaction and putative regulatory effect on the A-clade PP2C proteins from *Arabidopsis*, such as HAB1, PP2CA, and ABI1. An ABA-dependent inhibitory effect was found of ≈95% and 60–70% on HAB1 and ABI1/PP2CA activity, respectively (Fig. 1*B* and Fig. S2). The results with NtPYL4 correlated well, in general terms, with those with the *Arabidopsis* PYL4/PYL5. For instance, some Y2H interactions of PYL proteins with PP2C phosphatases did not depend on ABA, but the inhibition of PP2C activity did, both for NtPYL4 (Fig. 1*B* and 1*C*) and *Arabidopsis* PYL proteins (33). Taken together, these results indicate that NtPYL4 is a functional ABA receptor.

Constitutive *NtPYL4* Overexpression Alters the Tobacco Root Metabolome and Decreases Pyridine Alkaloid Accumulation. JA signal transduction cascades can usually be initiated throughout the whole plant, but in tobacco, alkaloid biosynthesis and its modulation by JAs is confined to the roots. To assess the role of

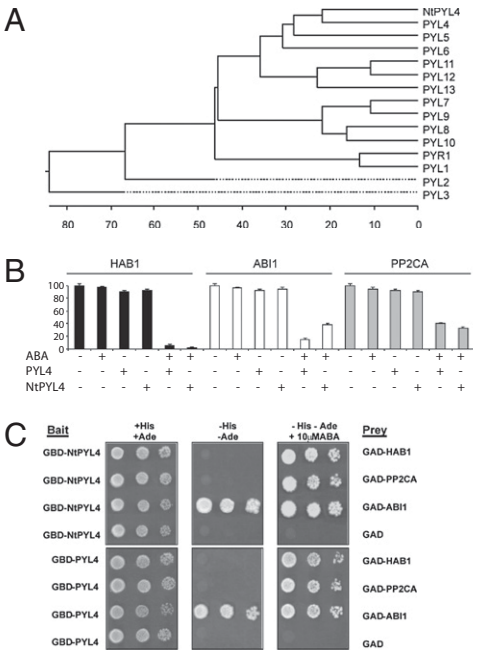

Fig. 1. The NtPYL4 protein is a functional ABA receptor. (*A*) Cladogram obtained from the ClustalW multisequence alignment. Numbers in the *x* axis indicate the amino acid substitutions (×100). (*B*) Inhibitory effect of NtPYL4 and PYL4 proteins on the phosphatase activity of *Arabidopsis* ABI1, HAB1, and PP2CA. Data are means ± SE from three independent experiments. Numbers in the *y* axis indicate percent PP2C activity compared with the control in absence of ABA, PYL4, or NtPYL4. (*C*) Interaction of NtPYL4 with *Arabidopsis* clade A PP2Cs in Y2H. Transformants were spotted on SD-Leu-Trp as a control for growth (+His+Ade). To investigate interaction of bait and prey, yeasts were dropped in serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) on selective media additionally lacking His and Ade, supplemented or not with ABA, as indicated.

NtPYL4 in the MeJA-mediated reprogramming of tobacco metabolism, we first analyzed *NtPYL4* expression in different organs of wild-type tobacco plants by quantitative real-time (qRT)-PCR (Fig. S3). In summary, (*i*) the *putrescine N-methyl transferase* (*PMT*) gene was exclusively expressed in roots, in accordance with its specific role in nicotine biosynthesis; (*ii*) the *quinolinate phosphoribosyltransferase* (*QPRT*) gene was expressed predominantly in roots, but also in leaves in accordance with its role in the conserved primary metabolic pyridine nucleotide cycle; (*iii*) induction by MeJA of *PMT* and *QPRT* occurred steadily over 24 h and was exclusively confined to the roots; and (*iv*) *NtPYL4* expression was detected both in leaves and roots in wild-type tobacco plants and was also modulated by MeJA in both organs (Fig. S3). In leaves, MeJA induced the expression of *NtPYL4* transiently, with a peak after 30 min, in contrast to roots, in which the *NtPYL4* expression transiently decreased with a maximum of fivefold reduction after 2 h of MeJA treatment (Fig. S3). Hence, the *in planta* expression pattern of *NtPYL4* differed qualitatively and quantitatively from that of the alkaloid biosynthetic genes *QPRT* and *PMT* (Fig. S3).

Second, gain-of-function experiments in tobacco hairy roots were set up. Eight transgenic and eight control tobacco hairy root clones were generated constitutively overexpressing *NtPYL4* and the *GUS* gene, respectively. Their metabolite profiles were compared by one-dimensional ^{1}H NMR, and overexpression of *NtPYL4* was confirmed by qRT-PCR. In all but one of the overexpression lines, the *NtPYL4* transcript levels were maximally twofold higher than those of the control roots (Fig. S4*A*). Raw ^{1}H NMR spectral data of *NtPYL4* and *GUS* root extracts were processed for multivariate data analysis to identify the differences between the sample sets, first by searching for sample group

clustering. Principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA) showed a good separation of the *NtPYL4* and *GUS* clusters, indicating that the metabolism was considerably altered in the *NtPYL4* root lines compared with the *GUS* control lines (Fig. S5*A*). To determine the variables (i.e., metabolite peaks) responsible for the group separations, OPLS-DA with a class label as variable was carried out on the same data set and overlaid with an OPLS analysis of the *NtPYL4* expression data.

This integrated analysis indicated that both primary and secondary metabolism were modulated as a result of *NtPYL4* overexpression (Fig. 2*A*, Fig. S5*B*, and Table S1). Most secondary metabolites with significantly lower concentrations in the *NtPYL4*-overexpressing lines than those in the control lines were identified as pyridine alkaloids, in particular nicotine, anatabine, and anatalline (−31%, −54%, and −42%, respectively). In contrast, phenylpropanoids accumulated more abundantly in the *NtPYL4*-overexpressing roots. Chlorogenic acid, the major phenylpropanoid that could be detected in tobacco hairy roots, increased on average with ≈27% in *NtPYL4* roots and other, minor phenylpropanoids, such as the 4-*O*- and 5-*O*-caffeoyl quinic acid isomers with ≈57%. Accumulation of amino acids such as glutamine and lysine, as well as of the nonprotein amino acid GABA, decreased in the *NtPYL4*-overexpressing roots by 35–72%. The *NtPYL4* roots also exhibited a substantially different pool of other primary metabolites, with 2- to 10-fold increased amounts in cytosine, formate, linoleic acid, and polyamine levels, for instance (Fig. 2*A*, Fig. S5*B*, and Table S1). Collectively, these findings indicate that *NtPYL4* overexpression substantially reprograms both primary and secondary metabolism in tobacco roots.

Constitutive *NtPYL4* Overexpression Alters the Tobacco Root Transcriptome and Down-Regulates the Expression of Nicotine Biosynthesis Genes. The metabolome analysis of transgenic lines suggested that *NtPYL4* overexpression affected diverse aspects of tobacco root metabolism. Therefore, we verified whether *NtPYL4* overexpression also influenced the tobacco root transcriptome. qRT-PCR analysis showed that the steady-state levels of the *PMT* and *QPRT* transcripts were lower in *NtPYL4*-overexpressing roots than those in control roots. *NtPYL4* and alkaloid biosynthesis transcript levels correlated inversely (Fig. S4 *B*–*E*), indicating that *NtPYL4* overexpression repressed pyridine alkaloid biosynthesis at the transcriptional level, in agreement with the observed decrease in alkaloid levels.

Because relatively little is known regarding the identity of the tobacco genes involved in most of the other metabolic pathways that were affected in *NtPYL4*-overexpressing roots, a nontargeted and genome-wide cDNA–amplified fragment length polymorphism (cDNA-AFLP)–based transcript profiling analysis was subsequently carried out to visualize the effects of *NtPYL4* overexpression on the tobacco root transcriptome. The quantitative accumulation patterns of 15,880 transcript tags were determined. In total, 131 transcript tags that were differentially expressed between control and *NtPYL4* roots could be unambiguously annotated, of which 42 (32%) corresponded to enzymes or transporters putatively involved in metabolism (Fig. S4*F*). Within this gene set, 29 transcript tags were up-regulated in at least three independent *NtPYL4* root lines, including a number of tags corresponding to proteins potentially involved in the synthesis of chlorogenic acid and other phenylpropanoids, such as 4-coumarate-CoA ligase, a putative hydroxycinnamoyl transferase, peroxidases, a MATE transporter that is closely related to the *Arabidopsis* FLOWER FLAVONOID TRANSPORTER (38), and a receptor kinase closely related to the *Arabidopsis THESEUS1* that acts as a cell wall integrity sensor and mediates ectopic lignin production in response to cellulose synthesis perturbations (39). Furthermore, a tag corresponding to 3-hydroxyisobutyrate dehydrogenase, an enzyme involved in valine, leucine, and isoleucine catabolism, was up-regulated, and the expression was altered (up or down) of several gene tags corresponding to enzymes involved in carbohydrate and

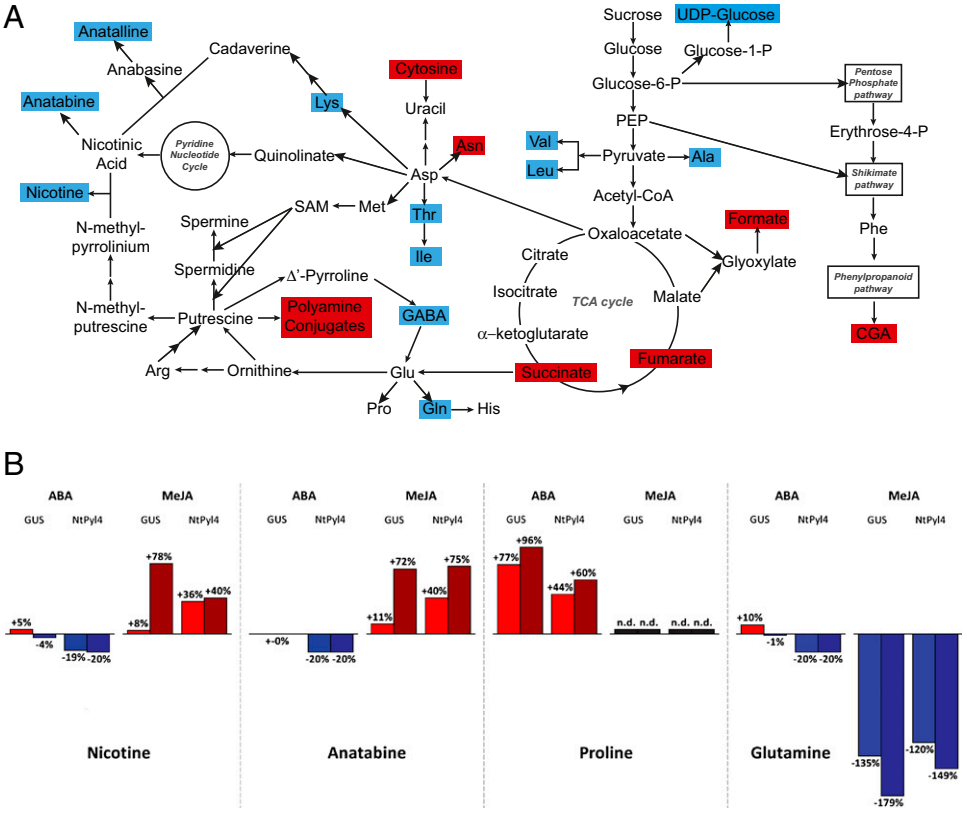

Fig. 2. *NtPYL4* overexpression reprograms metabolism in transgenic tobacco hairy roots. (*A*) Schematic representation of the effects of *NtPYL4* overexpression on tobacco root metabolism. Red and blue boxes show metabolites with increased and decreased accumulation levels, respectively, in *NtPYL4*-overexpressing tobacco roots in comparison with control roots. PEP, phospho*enol*pyruvate; SAM, *S*-adenosylmethionine. (*B*) Detected key metabolites affected by MeJA and ABA elicitation. Metabolite changes are illustrated at two time points (24 h and 48 h, left and right numbers, respectively) with colored bars (red and blue, positive and negative changes, respectively), of which the length represents the percentage change in an absolute scale compared with DMSO-treated samples. Values represent the mean of three biological repeats. Statistical significance was assessed by a two-tailed Student *t* test (Table S2).

lipid metabolism, which might account for the accumulation changes in primary metabolites in *NtPYL4* roots. Hence, the transcriptome analysis mirrors the NtPYL4-mediated reprogramming of both primary and secondary metabolism.

ABA Reduces Alkaloid Production in *NtPYL4*-Overexpressing Tobacco Roots. Exogenous ABA does not affect nicotine biosynthesis in *Nicotiana sylvestris* (40) and, to our knowledge, no other data have been reported that might support a possible link between ABA signaling and the regulation of alkaloid biosynthesis in tobacco. The identification of NtPYL4 as a functional ABA receptor and the effect of *NtPYL4* overexpression on the tobacco root metabolome and transcriptome prompted us to assess the functionality of the JA and ABA signaling cascades in *NtPYL4* root lines.

First, we determined the endogenous JA and ABA levels in control and *NtPYL4* root lines. For JA and its bioactive JA-Ile conjugate, variations were observed, but no clear difference between *GUS* and *NtPYL4* lines could be detected (Fig. S6). Hence, shifts in endogenous JA levels seemed not to be responsible for the reduced alkaloid levels in the *NtPYL4*-overexpressing roots. In contrast, ABA levels had increased in *NtPYL4* lines, but only by approximately twofold (Fig. S6), the possible impact of which is uncertain because drought stress, for instance, increases ABA levels by 10- to 40-fold (41). Nonetheless, a cumulative effect with the two- to fourfold overexpression of the *NtPYL4* ABA receptor might occur.

Second, the metabolic response of the transgenic tobacco hairy root lines to exogenous ABA and MeJA was profiled. Multivariate data analysis of the NMR spectra did not reveal marked general differences in MeJA response in terms of metabolite accumulation between *GUS* and *NtPYL4* root lines (Fig. 2*B* and Table S2). Furthermore, the MeJA induction of *PMT* and *QPRT* transcripts levels did not differ between the *GUS* and *NtPYL4* root lines, suggesting that *NtPYL4* overexpression did not directly interfere with JA signal transduction in tobacco hairy roots.

Treatment with ABA did not affect the biosynthesis of nicotine or other pyridine alkaloids in control tobacco root lines (Fig. 2*B* and Table S2). Overall, the separation between control root lines elicited by ABA and mock treated (DMSO) was small, and the accumulation trends varied only for a few metabolites [e.g., threonine (down) and proline (up)] (Fig. 2*B* and Table S2). In the *NtPYL4*-overexpressing hairy roots, the response to the ABA application was altered, reflected for instance by a reduced increase in proline accumulation (Fig. 2*B* and Table S2) and compromised root growth during the 48-h-long experiment (Fig. S7*A*). Expression of the ABA-responsive *WAPK* gene (42) was 4.2-fold higher in the absence of ABA in *NtPYL4* roots but markedly more decreased by ABA elicitation in *NtPYL4* than in the *GUS* roots (3.4-fold vs. 1.3-fold, respectively) (Fig. S7*B*). Expression of the *NtT172* gene, a tobacco homolog of the *Arabidopsis* HAB1 phosphatase and previously isolated in our cDNA-AFLP study as a MeJA-inducible gene (10), was induced with equal efficiency in *GUS* and *NtPYL4* tobacco root lines (Fig. S7*B*). *NtPYL4* expression was not affected by ABA treatment in any line (Fig. S7*B*). Notably, whereas ABA did not affect alkaloid biosynthesis in control roots, it was capable of reducing alkaloid biosynthesis in *NtPYL4*-overexpressing hairy roots (Fig. 2*B* and Table S2). In accordance with its effect on alkaloid accumulation, ABA treatment reduced *PMT* transcript levels in *NtPYL4* roots (2.3-fold vs. 1.3-fold in *GUS* roots) (Fig. S7*B*). Overall, these findings support an inhibitory role for the ABA signaling cascade in the control of tobacco alkaloid biosynthesis and confirm NtPYL4 as an ABA receptor.

PYL Loss of Function Alters the JA Response in Transgenic *Arabidopsis* Plants. To provide further support for the involvement of PYR/PYL/RCAR receptors in the ABA–JA interplay, we included *Arabidopsis* as a second experimental organism. First, we consulted Genevestigator (43) to verify whether the expression of the closest *Arabidopsis* homologs of *NtPYL4* [i.e., *PYL4* (At2g38310), *PYL5* (At5g05440), and *PYL6* (At2g40330)] was affected by MeJA treatment. Expression of all three *Arabidopsis PYL* genes was regulated by treatment with MeJA (https://www.genevestigator.com/ or http://www.arabidopsis.org/servlets/TairObject?type=locus&name=AT2G38310 for *PYL4*). *Arabidopsis PYL* expression could either be up- or down-regulated depending on the experimental setup of the transcriptome analysis and is thus probably subject to the context-dependent variability that is generally observed in JA transcriptional signatures (6).

Endorsed by this observation, we set out to study the JA response in a number of transgenic *Arabidopsis* lines with loss of function for *PYL* genes (Fig. 3 *A* and *B*). Both *pyl4* and *pyl5* knockout mutants were hypersensitive to JA after prolonged growth, reflected by a significant decrease in shoot biomass (Fig. 3*C*). In contrast, an opposite phenotype was observed for anthocyanin accumulation, which was less elicited by JA in *pyl4* and *pyl5* mutants than the wild-type Col-0 seedlings (Fig. 3*D*). Overall, these analyses supported that, as in tobacco, *Arabidopsis* PYL ABA receptors are involved in the crosstalk between the JA and ABA signaling pathways to regulate metabolism and growth.

Discussion

The negative role of PP2C proteins in ABA signaling and their regulation through PYR/PYL/RCAR proteins seems to occur in different plant species, from angiosperms to mosses (27). Here, we have identified a tobacco gene, *NtPYL4*, that codes for a protein belonging to the PYR/PYL/RCAR protein family of ABA receptors from *Arabidopsis*. Functional characterization demonstrated that NtPYL4 inhibits PP2C proteins in an ABA-dependent manner, and hence is a functional ABA receptor from tobacco. These findings point toward the existence of a conserved mechanism for ABA perception and signaling in plants.

Transcript profiling indicated that MeJA elicitation can modulate *NtPYL4* transcript levels in tobacco plants, at least transiently and either up- or downward depending on the organ and time after MeJA elicitation (10) (Fig. S3). Notably, MeJA elicitation of tobacco does not only affect expression of the ABA receptor gene *NtPYL4*, but also stimulates that of a gene encoding a tobacco PP2C (*NtT172*) with high sequence similarity to the *Arabidopsis* HAB1 phosphatase, both in cells (10, 36) and plants (Fig. S3). This concurrent transcriptional regulation of PYR/PYL/RCAR ABA receptor(s) and negative regulator PP2C(s) might serve as a fine-tuning mechanism in the monitoring of the JA and/or ABA responses and, ultimately, in balancing tradeoffs between growth and defense.

Tentatively, that in wild-type tobacco plants 24 h after MeJA elicitation, the expression of *NtPYL4* is either equal to the control level (in roots) or reduced twofold (in leaves), on the one hand, and that the expression of *NtT172* is induced (approximately fourfold in both leaves and roots), on the other hand, might imply that JAs shift the equilibrium to the inhibition of the ABA pathway throughout the whole plant, at least at the transcriptional level. In tobacco, this MeJA-regulated expression of the ABA perception system seemingly concurs with that of the genes involved in nicotine biosynthesis, suggesting a possible physiological link between these two events. Accordingly, genome-wide transcript and metabolite profilings demonstrated that the overexpression of *NtPYL4*, first, caused a genetic and metabolic reprogramming leading to decreased alkaloid accumulation, and second, altered the ABA responsiveness of tobacco roots. The latter was mostly reflected by the observation that exogenous ABA decreased pyridine alkaloid accumulation in *NtPYL4*-overexpressing roots, whereas the alkaloid levels in control roots remained constant after ABA application. Together, these findings append ABA to the list of hormones that

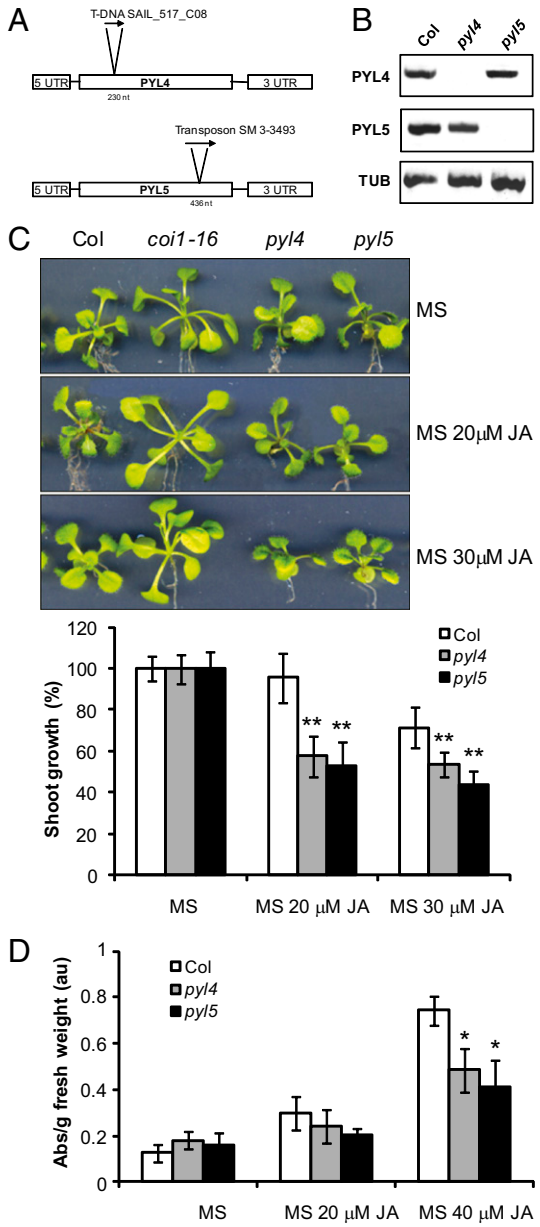

Fig. 3. Loss of function of *pyl4* and *pyl5* alters the JA response in transgenic *Arabidopsis* plants. (*A*) Schematic structure of the *pyl* loci showing the position of the T-DNA insertion. (*B*) RT-PCR analysis of seedlings from wild-type (Col) and *pyl* mutants with primer combinations diagnostic for the *PYL4*, *PYL5*, and *β-Tubulin* (*TUB*, At1g75780) transcripts. (*C*) Representative image and quantification of shoot growth in wild-type and *pyl* mutants after 22 d of growth on MS medium lacking or supplemented with JA. The JA-insensitive *coi1-16* mutant was included as a control for the JA treatment. The histogram represents the shoot growth relative to growth on control MS medium. Shoot weight (milligrams per seedling) was 11.38 ± 0.9, 10.01 ± 1.3, and 11.20 ± 1.6 for wild-type, *pyl4*, and *pyl5* plants grown on MS medium, respectively. Error bars indicate the SE ($n \geq 25$) from two different experiments. Statistical significance was determined by the Student *t* test. $^{**}P < 0.01$. (*D*) Anthocyanin accumulation in wild-type and *pyl* mutants after 22 d of growth on MS medium lacking or supplemented with JA. Absorbance values on the ordinate correspond to $(Abs_{530}\text{-}Abs_{657})$/gram of fresh shoot weight. Error bars represent the SE ($n = 4$). Statistical significance was determined by the Student *t* test. $^{*}P < 0.05$.

can crosstalk with JA in the regulation of the biosynthesis of alkaloids in tobacco and reveal that NtPYL4 is involved in the crosstalk between the hormonal signaling cascades.

Genevestigator (43) indicated that expression of the *Arabidopsis* genes of the *PYL4*/*PYL5*/*PYL6* branch can also be modulated by JAs (up or down), as well as in response to several elicitors, including wounding, flagellin, and syringolin treatment and infection with powdery mildew (*Blumeria graminis*) and *Pseudomonas syringae* (e.g., http://www.arabidopsis.org/servlets/TairObject?type=locus&name=AT2G38310), suggesting that crosstalk between the ABA, JA, and possibly other hormonal signaling pathways through particular PYR/PYL/RCAR family members in response to elicitors or stresses might be a conserved feature in the plant kingdom. Correspondingly, in transgenic *Arabidopsis* lines with *PYL4* or *PYL5* loss of function, the biomass and anthocyanin production in response to JA elicitation decreased, suggesting that PYL4/PYL5 might act to suppress JA-mediated growth inhibition and promote certain JA-mediated defense responses in wild-type *Arabidopsis*. Together, these results validate that altered *PYL* expression affects the JA response and that PYR/PYL/RCAR ABA receptors function in the crosstalk between the JA and ABA signaling pathways in different plant species.

A prominent physiological role of ABA concerns the regulation of the accumulation of osmocompatible solutes in response to dehydration (27), and a "multifaceted role" for ABA in biotic disease resistance has been proposed (25, 26). Therefore, PYL4 and/or other PYR/PYL/RCAR proteins might plausibly function as "reset buttons" through which hormonal signaling pathways (re)organize plant metabolism, for instance to divert metabolites from primary carbon and carbohydrate metabolism to amino acid metabolism. Indeed, as for the well-established role of JA in the modulation of plant metabolism (6, 8), the awareness of the interaction between ABA and primary and secondary metabolism is increasing rapidly (29, 44). Hence, ABA–JA crosstalk point(s) might act as elicitor response checkpoint(s) to control the stress-induced reprogramming of the plant metabolism, both quantitatively and/or qualitatively, for instance toward the production of particular bioactive or protective compounds needed to face particular biotic or abiotic stresses or to ensure an appropriate fitness-cost response when plants experience prolonged stress periods (25, 45).

Materials and Methods

PP2C Enzyme Assay. The *NtPYL4*-coding sequence was amplified by PCR and fused by Gateway recombination to the 6xHis tag in the pDEST17 vector (Invitrogen). Recombinant PYL4 and PP2C proteins were purified, and the PP2C phosphatase activity was measured in the presence or absence of the recombinant PYL4 proteins as described previously (33).

Y2H Assay. As baits, the coding sequences of *NtPYL4* and *PYL4* were amplified by PCR and fused by Gateway recombination to the GAL4 DNA-binding domain in the pGBT9 vector. As preys, a set of *Arabidopsis* A-clade PP2C proteins was amplified by PCR and cloned into the pCR8/GW/TOPO vector. Subsequently, the full-length ORFs were excised with restriction enzymes and fused to the GAL4 activation domain in the pGAD424 vector. The Y2H assay was done as previously described (32).

Generation and Elicitation of Tobacco Hairy Root Cultures. The full-length *NtPYL4* and *β-D-glucuronidase* (*GUS*) ORFs were put under the control of the constitutive pCaMV-35S promoter by Gateway recombination in the pK7WG2D vector and subsequently introduced into the *Agrobacterium rhizogenes* strain LBA9402 that was used to generate hairy roots from *N. tabacum* cv. Basma Xanthi leaves, as previously described (36). Well-growing root clones were individually cultivated in liquid medium for 3 wk. For elicitation, the culture medium (20 mL) was replaced by a fresh aliquot containing either 50 μM (+)-ABA (Duchefa), 50 μM MeJA (Duchefa), or the corresponding amount of DMSO (the solvent). Samples were taken after 24 h and 48 h and immediately shock-frozen in liquid nitrogen.

Gene Expression Analysis. qRT-PCR expression analysis of roots and leaves from wild-type tobacco plants and transgenic tobacco hairy roots was carried out as previously described (36). Primers were designed according to sequence data from *NtPYL4* (AJ966358), *NtT172* (CQ809147), *NtPMT4*

(AF126812), *NtQPRT* (AB038494), *WAPK* (AF032465), and *GRP1* (EU569289). *β-ATPase* (U96496) and *ST30* (AM991691) were used as the reference genes.

cDNA-AFLP transcript profiling on transgenic tobacco hairy roots was carried out as previously described (46). Gene tags of which the expression values had a coefficient of variation ≥0.5 were considered as differentially expressed. On the basis of this cutoff value, together with visual inspection of the cDNA-AFLP gels, differentially expressed gene tags were selected for further analysis. Cluster and sequence analysis were done as previously described (47).

Hormone and Metabolite Profiling in Tobacco Hairy Root Lines. The tobacco root metabolite profiles were established by NMR analysis as described in detail in *SI Materials and Methods*. The levels of ABA and JA hormones were analyzed essentially as previously described (48), with some modifications (*SI Materials and Methods*).

Analysis of Transgenic and Mutant *Arabidopsis* Plants. *pyl4* and *pyl5* knockout mutants were identified from Syngenta Arabidopsis Insertion Library (SAIL) T-DNA and JIC SM collections (donor stock numbers SAIL_517_C08 and SM 3-3493, respectively). For phenotypic analysis, Columbia wild-type and *pyl* knockouts were grown in vitro as previously described (49). After 22 d of growth on Murashige and Skoog (MS) medium with or without JA (Sigma-Aldrich), seedlings were individually scored for shoot weight and subsequently pooled in batches to determine anthocyanin content (17).

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