Metabolic Engineering of Isoprenoid Biosynthesis in *Arabidopsis* for the Production of Taxadiene, the First Committed Precursor of Taxol

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Abstract: Paclitaxel (Taxol) is a widely used anticancer isoprenoid produced by the secondary metabolism of yew (*Taxus* sp.) trees. However, only limited amounts of Taxol or related metabolites (taxoids) can be obtained from the currently available sources. In this work we have taken the first step toward genetically engineering the biosynthesis of taxoids in angiosperms. The first committed step in Taxol biosynthesis is the production of taxadiene from geranylgeranyl diphosphate (GGPP), catalyzed by the plastid-localized enzyme taxadiene synthase (TXS). A recombinant *T. baccata* TXS lacking the putative plastid targeting peptide and fused to a C-terminal histidine (His) tag was shown to be enzymatically active in *Escherichia coli*. Constitutive production of the full-length His-tagged enzyme in *Arabidopsis thaliana* plants led to the accumulation of taxadiene and concomitant growth retardation and decreased levels of photosynthetic pigment in transgenic plants. Although these phenotypes may derive from a toxic effect of taxadiene, the lower accumulation of endogenous plastid isoprenoid products such as carotenoids and chlorophylls in transgenic plants also suggests that the constitutive production of an active TXS enzyme might alter the balance of the GGPP pool. Induction of transgene expression using a glucocorticoid-mediated system consistently resulted in a more efficient recruitment of GGPP for the production of taxadiene, which reached levels 30-fold higher than those in plants constitutively expressing the transgene. This accomplishment illustrates the possibility of engineering the production of taxoids and other GGPP-derived isoprenoids in crop plants despite the constraints associated with limited knowledge with regard to regulation of GGPP availability.
Keywords: Arabidopsis; glucocorticoid; isoprenoids; paclitaxel; taxadiene; geranylgeranyl diphosphate

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INTRODUCTION

Isoprenoids (also called terpenoids) are the most functionally and structurally diverse group of plant metabolites. They are present in all organisms but are especially abundant in plants, with more than 30,000 compounds reported to date (Chappell, 1995; McGarvey and Croteau, 1995; Croteau et al., 2000). Many isoprenoids have primary roles in membrane architecture (phytosterols), protein modification (dolichol, prenyl groups), respiration (ubiquinones), photosynthesis (carotenoids, chlorophylls, plastoquinones), and regulation of growth and development (gibberellins, abscisic acid, cytokinins, brassinosteroids). The largest variety of isoprenoids, however, are secondary metabolites that function in protecting plants against herbivores and pathogens, in attracting pollinators and seed-dispersing animals, and as allelochemicals that influence competition among plant species (Croteau et al., 2000). Each plant species synthesizes a specific array of isoprenoid secondary metabolites, mostly produced only in wild or semi-wild plant species. The diterpenoid paclitaxel is one of these. (Paclitaxel is the generic name for Taxol, which is a registered trademark of Bristol-Myers Squibb. Because of the far greater familiarity with the word Taxol, we will use it here instead of paclitaxel.)

Taxol is synthesized in the bark of yew trees such as *Taxus brevifolia*, which are very slow growing and sparsely distributed. In addition, the amount of Taxol produced in the yew bark is low and its harvest is destructive. Because Taxol is one of the most potent chemotherapeutic agents known against a range of cancers (Arbuck and Blaylock, 1995; Jennewein and Croteau, 2001), its demand greatly exceeds supply from its natural source. Alternative approaches for obtaining Taxol include total synthesis of the drug, which is costly and low yielding (Kingston, 1994), and semisynthesis from abundant, more readily available biosynthetic precursors such as 10-deacetylbaccatin III and baccatin III (Ketchum and Croteau, 1998). Although semisynthesis is

the main procedure for commercial production of Taxol, purification of the precursors from renewable foliage and other tissues of plantation-grown *Taxus* species requires substantial effort. The production of Taxol and related taxane diterpenoids (taxoids) in *Taxus* cell culture systems appeared to be a promising strategy, but the yields have been unstable and high production levels difficult to sustain (Jennewein and Croteau, 2001; Ketchum and Croteau, 1998). Intensive efforts have been directed toward finding other means of producing and synthesizing Taxol analogs with increased activity and solubility. For the foreseeable future, however, an efficient and affordable supply of Taxol and taxoids should rely on alternative biological production systems (Jennewein and Croteau, 2001; Walker and Croteau, 2001). Because no routine transformation systems are currently available for *Taxus* species, we have explored the possibility of transferring the pathway to fast-growing plant species that are easier to genetically manipulate as an alternative to currently available methods for the production of taxoids.

The first step in the biosynthesis of Taxol (Fig. 1A) is the production of the committed intermediate taxadiene [taxa-4(5),11(12)-diene] by cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate (GGPP). This reaction is catalyzed by taxadiene synthase (TXS), a plastid-localized enzyme (Koepp et al., 1995). After taxadiene synthesis, it takes about 12 enzymatic steps (including hydroxylations and acylations of the taxane skeleton) to synthesize Taxol, but fewer are required to produce the advanced taxoid intermediate 10-deacetylbaccatin III (Fig. 1A), the major current source for semisynthesis of Taxol (Jennewein and Croteau, 2001; Walker and Croteau, 2001). Although the microsomal cytochrome P450 monooxygenase that hydroxylates taxadiene to produce the next intermediate of the pathway has not yet been cloned, several of the genes encoding the remaining biosynthetic enzymes have been identified (Walker and Croteau, 2001). The GGPP substrate used by TXS appears to be derived mostly from the methylerytritol 4-phosphate (MEP) pathway (Eisenreich et al., 1996; Palazon et al., 2003), which synthesizes the prenyl diphosphate precursors for the production of isoprenoids in plastids (Rodriguez-Concepcion and Boronat, 2002). Plastidial GGPP is also a precursor for the biosynthesis of phytohormones and photosynthesis-related isoprenoids in plants (Fig. 1B). In this study we show that the production of taxadiene and potentially downstream taxoids can be implemented in transgenic plants despite the problems derived from the interaction between endogenous and transferred GGPP-consuming pathways.

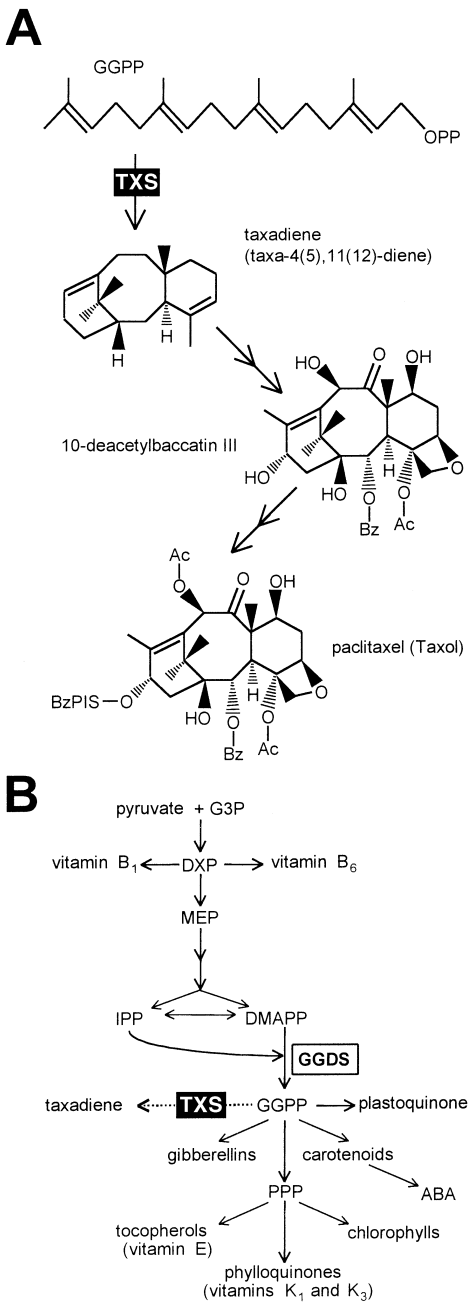

Figure 1. Taxol biosynthetic pathway. (A) TXS, taxadiene synthase; Ac, acetyl group; Bz, benzoyl group; BzPIS, *N*-benzoyl-3-phenylisoserine side chain. (B) Outline of GGPP-derived isoprenoid biosynthesis in plant cells. G3P, glyceraldehyde 3-phosphate; DXP, deoxyxylulose 5-phosphate; MEP, methylerythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; PPP, phytyl diphosphate; ABA, abscisic acid; GGDS, GGPP synthase.

MATERIALS AND METHODS

cDNA Cloning and Sequence Analysis

Total RNA was isolated from *T. baccata* cultured cells as described by Wang et al. (2000), and cDNA was synthesized with a cDNA synthesis system (Gibco-BRL). Primers TXS-1F (5′-ATGGCTCAGCTCTCATTTAATG-3′; the translation start codon is underlined) and TXS-7R (5′-TGCCAATACAATAATAAGTC-3′, annealing on the 3′-UTR) were designed after very highly conserved sequences in the *TXS* cDNAs from *T. brevifolia* (Accession No. U48796) and *T. chinensis* (AY007207) and used to amplify a *T. baccata* cDNA encoding a full-length protein with

homology to TXS (AY424738). The blunt-ended polymerase chain reaction (PCR) product obtained with *Pfu* DNA polymerase (Promega) was cloned into *Sma*I-digested pBluescript SK$^+$ (Stratagene) to generate plasmid pBS-TXS. Sequencing of the cloned cDNA was carried out with an ABI-Prism BigDye Terminator Cycle Sequencing kit, v2.0 (PE Biosystems). Sequence analyses were performed using GCG 9.0 software (Genetics Computer Group, Inc.) and the available web resources (www.expasy.ch and www.cbs.dtu.dk/services/ChloroP/).

Expression in *E. coli*

The RGS(6xH) epitope was cloned in-frame with the C-terminus of the predicted protein after amplifying the entire coding region of the *TXS* cDNA by PCR with *Pfu* DNA polymerase (Promega) and primers TXS-1F and TXS-6R-His (5′-TCACGTG**TCA***GTGATGGTGATGGTGATGC-GATCCTCT*TACTTGAATTGGGTCAATATAAAC-3′; the sequence annealing with the *TXS* cDNA is underlined). Primer TXS-6R-His was designed to only eliminate the stop codon and incorporate in its place a sequence encoding the epitope residues (in italics), a new stop codon (in bold), and a *Pml*I restriction site. The PCR product was cloned into *Sma*I-digested pBluescript SK$^+$ to generate plasmid pBS-TXS-His. A *Nco*I site was subsequently incorporated into the *TXS* cDNA cloned into pBS-TXS-His by PCR using primer TXS-*Nco*I-180F (5′-GTCGTAACCATGGGCAG-TAGCAC-3′; the *Nco*I site is underlined) to generate a new ATG codon 60 triplets downstream of the original translation start site. A *Nco*I–*Eco*RI cDNA fragment encoding the predicted mature TXS protein (without the predicted plastid targeting sequence) was cloned into the same sites of the expression vector pET23 (Novagen) for expression in *E. coli*. The resulting pET-ΔTXS-His plasmid was used together with pUBS520-*argU*, which encodes the bacterial minor tRNA(Arg) species to decode the plant-abundant codons AGA and AGG (Brinkmann et al., 1989) to cotransform BL21(DE3) cells. Protein expression was induced with 0.2 m*M* isopropyl-beta-D-thiogalactopy ranoside (IPTG) at 28°C and monitored by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. The recombinant His-tagged TXS protein was purified on a Hi-Trap chelating column (Amersham Pharmacia) and eluted with 50 m*M* Tris-HCl (pH 7.5), 100 m*M* NaCl, and 150 m*M* imidazol, following the manufacturer's recommendations. Analysis of this fraction by SDS-PAGE and Coomassie staining showed that >90% of the eluted protein corresponded to His-tagged TXS.

Expression in Plants

A 2.6-kb cDNA fragment encoding the full-length TXS protein fused to the RGS(6xH) epitope was isolated from pBS-TXS-His after digestion with *Nco*I and *Pml*I. The resulting fragment was gel-purified and subcloned into the *Nco*I and *Sma*I sites of pGFP-MRC (Rodriguez-Concepcion et al., 1999) to generate plasmid pGFP-TXS-His. Digestion of this plasmid with *Pst*I and *Xba*I (the *Xba*I site was blunt-ended with Klenow before digestion with *Pst*I) released the *TXS* cDNA fused to the CaMV *35S* promoter and a TEV enhancer. The resulting fragment was subcloned into the *Pst*I and *Pml*I sites of the pCAMBIA 1303 vector (AF234299) to generate plasmid pCA-TXS-His. A fragment without the CaMV *35S* promoter was obtained after digestion of pGFP-TXS-His with *Eco*RI and *Xba*I (the *Eco*RI site was blunt-ended with Klenow before digestion with *Xba*I) and cloned into pTA7002 (Aoyama and Chua, 1997) digested with *Xho*I, blunt-ended with Klenow, and digested with *Spe*I to generate plasmid pTA-TXS-His. Constructs pCA-TXS-His and pTA-TXS-His were delivered to the *Agrobacterium tumefaciens* strain C58C1 (pGV2260) and used to transform *Arabidopsis* plants (ecotype Columbia-3) by floral dipping (Clough and Bent, 1998). Transgenic plants were selected on Petri dishes with solid Murashige–Skoog (MS) medium supplemented with 50 μg/mL hygromycin (Carretero-Paulet et al., 2002). Homozygous T_3 plants were selected and used for the experiments reported in this work. Induction with dexamethasone (DEX; Sigma) was carried out either by supplementing MS plates with 30 μ*M* DEX (prepared from a 30 m*M* stock solution in ethanol) or by spraying plants with a solution containing 30 μ*M* DEX and 0.1% Tween-20 (Sigma).

Immunoblot Analyses

Plant protein extracts were then prepared as described by Carretero-Paulet et al. (2002). Aliquots of 50 μg of protein were separated on 8% acrylamide gels and either stained with Coomassie blue to monitor loading or electrotransferred to Hybond-P PVDF membranes (Amersham), as described by Rodriguez-Concepcion et al. (2001). The recombinant His-tagged TXS protein was detected with a 1:2000 dilution of an anti-RGS(6xH) polyclonal antibody (Qiagen), as described by Carretero-Paulet et al. (2002).

Pigment Measurement

Plastidial isoprenoid pigments were extracted and separated by high-performance liquid chromatography (HPLC) on a Waters Alliance 2690 system using a reverse-phase YMC 5-μm (250 × 4.6 mm) C30 column, as described by Fraser et al. (2000b). Eluting compounds were monitored using a photodiode array detector (Model 2996, Waters). Canthaxanthin was used as an internal standard for quantification. Peak areas of total chlorophylls and carotenoids at 450 nm were determined using MILLENIUM v3.2 software (Waters).

Determination of Taxadiene

In vitro TXS activity was determined as described by Hezari et al. (1995) with the following modifications. Ten micrograms of His-tagged TXS protein purified by immobilized metal-ion affinity chromatography (IMAC), as de-

scribed earlier, was incubated at 30°C for 12 h in the presence of 25 m*M* HEPES (pH 8), 50 μ*M* GGPP (Sigma), and 1 m*M* $MgCl_2$ in a final volume of 500 μL. After incubation, 1 mL of hexane was added and the reaction mixture vigorously vortexed. After centrifugation to separate phases, the hexane layer was collected and passed through a glasswool-plugged Pasteur pipette filled with silica gel with an average pore diameter of 25 Å (Sigma), overlaid with anhydrous $MgSO_4$. This step was repeated twice. The hexane extracts were collected and concentrated before gas chromatography–mass spectrometry (GC-MS) analysis, which was performed on a Trace-DSQ system (Thermo Finnigan) using a 25-m fused silica capillary HP-5 column with a 200-μm diameter and 0.5-μm film thickness. Samples were separated using splitless injection (250°C) with temperature programming to 320°C at 8°C/min and helium as a carrier gas. Electron impact spectra were recorded at 70 eV. For taxadiene quantification in plants, tissues were frozen in liquid nitrogen, ground to a fine powder, and mixed with 0.5 ng/mL nonadecane (Sigma) in hexane (3 mL/g of fresh tissue). The mixture was shaken overnight at 4°C and the hexane fraction was then passed through a column similar to that described earlier but surmounted by a top layer of activated charcoal. The eluted hydrocarbon fraction free of oxygenated metabolites was used for GC-MS analysis, as described previously.

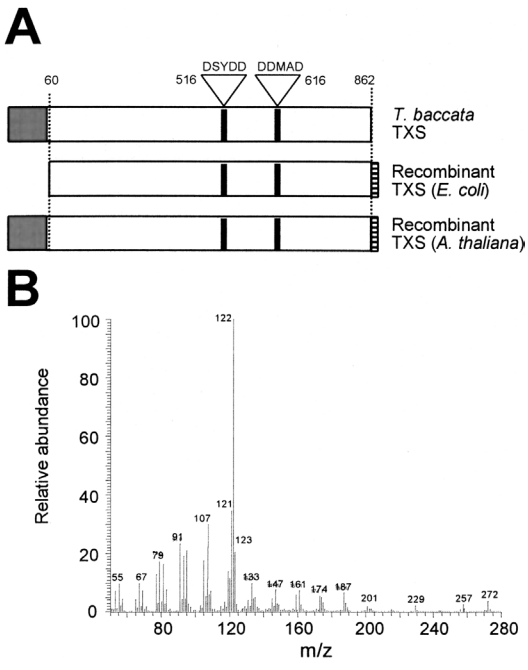

Figure 2. TXS derivatives and taxadiene mass spectrum. (A) Schematic map of the TXS proteins used in this study. The signal peptide predicted to target the protein to plastids by the ChloroP algorithm (Emanuelsson et al., 1999) is represented in gray, the typical terpene synthase divalent metal ion–substrate complex binding motifs (Wildung and Croteau, 1996) are indicated with black bars, and the C-terminal RGS(6xH) tag is shown as a hatched box. (B) Mass spectra of the product of the truncated recombinant TXS enzyme produced in *E. coli* using GGPP as a substrate. Identification of the mass fragmentation pattern as that of taxa-4(5),11(12)-diene is based on comparison to published data (Wildung and Croteau, 1996; Williams et al., 2000).

RESULTS AND DISCUSSION

Cloning of a *T. baccata* cDNA Encoding Taxadiene Synthase

An in vitro culture of Taxol-producing *T. baccata* cells was used as a source for the isolation of a cDNA encoding TXS. Reverse transcript (RT)-PCR with primers designed from highly conserved regions of cloned TXS sequences from *T. chinensis* and *T. brevifolia* successfully amplified a *T. baccata* cDNA sequence with a 2589-bp open reading frame encoding a predicted protein of 862 residues and 98 kDa. The deduced full-length protein showed homology to the previously cloned TXS enzymes (98.8% and 97.7% similarity to *T. chinensis* and *T. brevifolia* TXS proteins, respectively). The putative *T. baccata* enzyme also displayed two conserved motifs, likely involved in metal ion–substrate complex binding (Wildung and Croteau, 1996), and a putative plastidial transit peptide predicted to be cleaved at position 59 by the ChloroP algorithm (Emanuelsson et al., 1999) (Fig. 2A). Indeed, earlier studies have reported that the mature TXS enzyme is a plastid-localized monomeric protein of about 79 kDa (Hezari et al., 1995), which suggests that the cleavage of the plastid signal peptide likely occurs downstream of the predicted position. To ascertain the proposed function of the cloned protein, *Escherichia coli* cells were transformed with a construct expressing the predicted mature TXS enzyme fused to a RGS(6xH) tag (Fig. 2A). The 60 N-terminal residues likely involved in plastid targeting were removed to optimize expression of high levels of active protein (Williams et al., 2000), whereas the RGS(6xH) epitope was added to monitor production of the protein with a specific antibody and to facilitate the purification of the recombinant enzyme by IMAC (Huang et al., 1998). In vitro activity assays with the purified protein showed that it was able to convert GGPP into a compound identified as taxadiene by GC-MS (Fig. 2B), thus confirming that the identified *T. baccata* cDNA encodes a functional TXS. Furthermore, we found that neither the truncation of the predicted plastid targeting sequence nor the addition of the C-terminal RGS(6xH) tag prevents enzymatic activity.

Constitutive Production of Taxadiene Correlates With Deleterious Effects in *Arabidopsis*

Taxadiene has been synthesized in *E. coli* cells cotransformed with constructs to express TXS and GGPP synthase (an activity absent in the bacterium) (Huang et al., 2001). However, implementation of further enzymatic steps for the production of downstream taxanes in bacterial cells may be prevented by the need to express the required plant microsomal cytochrome P450 monooxygenases in an enzymatically active form. In addition, there are metabolic

constraints mostly derived from the need to channel a limited pool of bacterial precursors to GGPP biosynthesis, which may compromise the production of Taxol precursors (Huang et al., 2001). A similar scenario has been described for the production of carotenoids, another group of GGPP-derived isoprenoids (Fig. 1B) in *E. coli* (a non-carotenogenic bacteria) (Farmer and Liao, 2000; Sandmann, 2001). We therefore reasoned that plants might be a better system to engineer the production of taxoids. To overexpress TXS in *Arabidopsis* a chimeric cDNA encoding the full-length enzyme from *T. baccata* fused to a C-terminal RGS(6xH) tag (Fig. 2A) was cloned into the pCAMBIA 1303 vector under the transcriptional control of the cauliflower mosaic virus *35S* promoter (*35S:TXS* construct). Homozygous T_3 plants from seven independent *35S:TXS* lines were selected and used for immunoblot analysis with an anti-RGS(6xH) antibody, revealing the presence of a ca. 80-kDa protein in six of them, which was absent from wild-type plants and homozygous control plants (CC) transformed with the unmodified pCAMBIA 1303 vector (Fig. 3A). The size of the recognized protein was consistent with previous reports (Hezari et al., 1995) suggesting a plastidial localization of the enzyme. In agreement, the recombinant protein was found in the organellar fraction in subcellular fractionation experiments (data not shown). Lines C2 and C3, which showed the highest levels of recombinant protein (Fig. 3A), as well as the CC control line, were selected for further analyses. Figure 3B shows that the recombinant RGS(6xH)-tagged protein produced by C2 was active in producing a new compound in *Arabidopsis*, which was identified as taxadiene by GC-MS. Quantification of taxadiene in C2 and C3 plants was accomplished using nonadecane as an internal standard (Fig. 3B). Similar levels of taxadiene (around 20 ng/g dry weight) were produced in seedlings and leaves from both *35S:TXS* transgenic lines (Table I). We observed, however, that taxadiene accumulation in *35S:TXS* plants was correlated with a reduced hypocotyl length in dark-grown seedlings (Table I), retarded growth and flowering, and a pale, bleached phenotype (data not shown) due to reduced levels of chlorophylls and carotenoids compared with untransformed plants (Table I). One explanation for these phenotypes might be a toxic effect of taxadiene accumulation in plant cells. An alternative possibility is that the constitutive production of an active TXS enzyme might interfere with endogenous balance in the production of plastidial isoprenoids, such as the diterpenoid hormones gibberellins and carotenoids and the side chain of chlorophylls (see Fig. 1B). Similarly, dwarf tomato and tobacco plants were generated by constitutive overexpression of phytoene synthase, the enzyme catalyzing the conversion of GGPP into phytoene, which is the first committed step in the biosynthesis of carotenoids (Busch et al., 2002; Fray et al., 1995). In these plants, the synthesis of gibberellins was compromised because the GGPP precursor required for their synthesis was channeled to the carotenoid pathway by phytoene synthase (Fray et al., 1995) (see Fig. 1B). The affinity of *T. brevifolia* TXS for GGPP (K_m [GGPP] = 3 μM) (Hezari et al., 1995) is similar to that reported for other plant enzymes that utilize GGPP as substrate to catalyze the formation of the first committed steps of pathways leading to the production of carotenoids (phytoene synthase; 5 μM) (Fraser et al., 2000a) and the side chain of plastoquinone (solanesyl diphosphate synthase; 1.6 μM) (Hirooka et al., 2003). However, the relatively small amounts of taxadiene formed suggests that the recombinant TXS might not be channeling significant amounts of GGPP away from the biosynthesis of other plastidial isoprenoids. We propose that the presence of a non-native, GGPP-consuming enzyme such as TXS in *Arabidopsis* might interfere with the

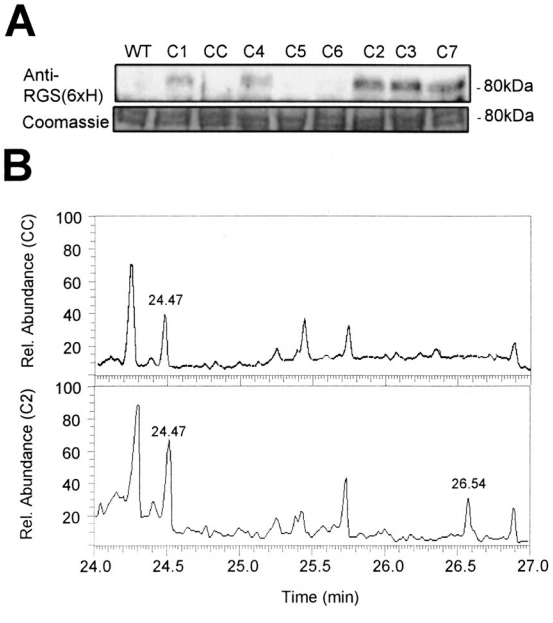

Figure 3. Constitutive expression of TXS in transgenic *Arabidopsis thaliana*. (A) Immunoblot analysis of extracts from 2-week-old seedlings of seven independent *35S:TXS* lines. A vector control line (CC) and the Columbia-3 wild-type (WT) were also analyzed with a commercial anti-RGS(6xH) antibody. (B) GC-MS analysis (total ion count) of the hexane extracts from CC (upper panel) and C2 (lower panel) leaves. Retention times of the nonadecane standard (24.47) and taxadiene (26.54) are indicated.

Table I. Characterization of transgenic plants constitutively overexpressing TXS.[a]

	Line		
	CC	C2	C3
Seedlings			
Taxadiene (ng/g DW)	—	23.45 ± 2.76	19.21 ± 1.31
Hypocotyl length (mm)	10.89 ± 2.18	8.02 ± 1.64	7.77 ± 1.48
Carotenoids (mg/g FW)	0.81 ± 0.02	0.62 ± 0.04	0.64 ± 0.01
Chlorophylls (mg/g FW)	1.20 ± 0.03	0.75 ± 0.04	0.87 ± 0.04
Leaves			
Taxadiene (ng/g DW)	—	24.64 ± 4.10	17.52 ± 0.56
Carotenoids (mg/g FW)	1.47 ± 0.16	1.03 ± 0.15	1.03 ± 0.10
Chlorophylls (mg/g FW)	2.10 ± 0.15	1.50 ± 0.03	1.58 ± 0.07

[a]DW, dry weight; FW, fresh weight.

regulation of the availability of intermediates for production of endogenous plastidial isoprenoids, many of which are key for plant growth and development.

Induction of TXS Expression in Mature Leaves Results in a Dramatic Increase in Recombinant Protein Levels and Taxadiene Production

An alternative strategy for the production of taxadiene in transgenic *Arabidopsis* plants consisted in the utilization of an inducible system to control expression of the transgene. We reasoned that this approach could result in higher yields of taxadiene by expressing the recombinant TXS when GGPP levels are less critical for plant growth and development. The cDNA encoding the full-length enzyme from *T. baccata* fused to a C-terminal RGS(6xH) tag (Fig. 2A) was cloned into the pTA7002 vector, which contains a two-component glucocorticoid system that utilizes the regulatory mechanism of steroid hormone receptors from mammals, allowing tight regulation of transgene expression in plants (Aoyama and Chua, 1997). *Arabidopsis* plants transformed with this construct (*TA:TXS*) were expected to express only recombinant TXS after glucocorticoid treatment. Indeed, immunoblot analysis using the anti-RGS(6xH) antibody of seedlings from five independent *TA:TXS* lines revealed the presence of a cross-reacting band in all of them only when the synthetic glucocorticoid dexamethasone (DEX) was added to the medium in which they were germinated and grown (Fig. 4A). The protein was absent from wild-type plants and control plants transformed with the unmodified vector (CI), as well as from non-induced *TA:TXS* transgenic plants, confirming that expression of the transgene could be regulated efficiently. Homozygous T_3 plants of the line I2, which showed high levels of the recombinant protein only in the presence of DEX (Fig. 4A), were selected for further analyses. As expected, in I2 seedlings germinated and grown on medium supplemented with DEX, the accumulation of recombinant enzyme, the production of taxadiene, and the depletion of isoprenoid pigments were very similar to those described for *35S:TXS* seedlings (data not shown), because in both cases the plants continuously express the *TXS* transgene. To optimize the conditions for taxadiene production, we studied the time-course of TXS accumulation after DEX-mediated induction of transgene expression in mature leaves, which show an active MEP pathway (Carretero-Paulet et al., 2002; Estevez et al., 2000), and yet the production of plastidial GGPP-derived end-products is less critical than that in developing seedlings and young leaves. Samples of mature leaves from I2 plants at the vegetative stage were collected at 24-h intervals after DEX spraying and used for immunoblot analysis with the anti-RGS(6xH) antibody. As shown in Figure 4B, a very strong DEX-dependent induction of TXS production was observed, reaching the highest levels 2 days after spraying and then progressively declining. At 4 days after induction, the levels of recombinant enzyme in the induced *TA:TXS* plants were still higher than those found in leaves from the *35S:TXS* lines C2 and C3 (Fig. 4B). Leaves from I2 plants were collected 2 days after spraying with DEX and used to monitor the production of taxadiene, which was much higher than that observed in plants constitutively expressing TXS (compare Fig. 4C with Fig. 3B). Quantification of taxadiene in these samples showed levels of up to 600 ng of taxadiene per gram of dry weight, a ca. 30-fold increase compared with that measured in seedlings and young leaves constitutively expressing the transgene (Table I). We believe that this occurs because the production and accumulation of very high levels of active TXS enzyme in a relatively short period can more efficiently recruit the available GGPP substrate for taxadiene biosynthesis.

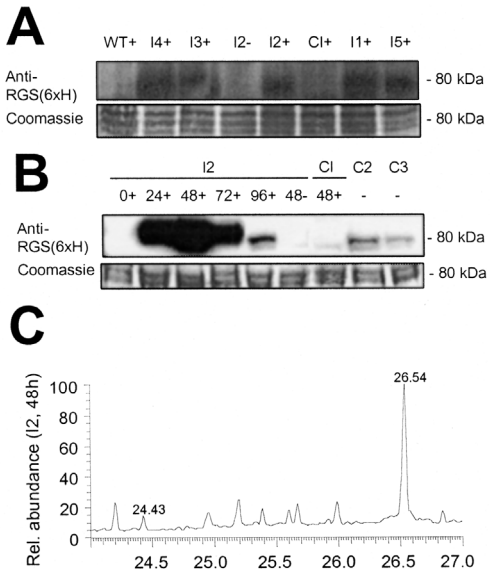

Figure 4. Inducible expression of TXS in transgenic *Arabidopsis thaliana.* (A) Immunoblot analysis with the anti-RGS(6xH) antibody of protein extracts from 2-week-old seedlings of five independent *TA:TXS* lines germinated and grown on MS plates either supplemented (+) or not (−) with DEX. A vector control line (CI) and the Columbia-3 wild-type (WT) grown on DEX-containing plates were also analyzed. (B) Mature leaves of the *TA:TXS* line I2 were sprayed either with DEX (+) or with a mock solution (−), and samples were collected after the indicated times (in hours) for immunoblot analysis with the anti-RGS(6xH) antibody. Protein extracts from leaves of the vector control line (CI) collected 48 h after DEX treatment and from untreated leaves of C2 and C3 plants (harboring the *35S:TXS* transgene) were also analyzed. (C) GC-MS analysis (total ion count) of the hexane extracts from I2 mature leaves collected 48 h after spraying with DEX. Retention times of the nonadecane standard (24.43) and taxadiene (26.54) are indicated.

Our results demonstrate for the first time that functional steps of the Taxol biosynthesis pathway can be reproduced in plants other than *Taxus*. We have also shown that significant increases in the production of taxadiene can be accomplished by selecting the temporal and spatial coordinates of TXS expression, establishing a platform technology for future metabolic engineering approaches aimed at production of taxoids (and likely other GGPP-derived isoprenoids) in crop plants. Alternatives to the strategy described herein to increase the availability of the

GGPP substrate include metabolic engineering of the MEP pathway for production of higher levels of this isoprenoid precursor. For instance, overexpression of deoxyxylulose 5-phosphate synthase (the first enzyme of the MEP pathway) results in enhanced accumulation of plastidial isoprenoid end-products, suggesting that this activity is limiting for production of their precursors in *Arabidopsis* (Estevez et al., 2001). In addition, the transformation of other plants with constructs to target the expression of the *TXS* gene to tissues or organs with a more active secondary metabolism and higher production of GGPP-derived isoprenoids (such as tomato fruit during ripening) might permit a further increase in taxadiene production and may facilitate harvesting without disturbing the endogenous metabolic balance. Future efforts should also be directed at implementation of other biosynthetic steps for transgenic plants in the production of taxoids that could be then purified and used in semisynthesis approaches. In addition, the expression in such transgenic plants of chimeric genes generated by in vitro evolution or shuffling strategies might contribute to generation of structurally novel taxoids with a greater range and potency of anticancer activity, increased bioavailability, and decreased side effects.

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