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JASMONATE MODULATES DEVELOPMENT- AND LIGHT-REGULATED ALKALOID BIOSYNTHESIS IN *CATHARANTHUS ROSEUS**

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

FELIPE A. VÁZQUEZ-FLOTA and VINCENZO DE LUCA†

Institut de Recherche en Biologie Végétale, Département de Sciences Biologiques, Université de Montréal, 4101 Rue Sherbrooke Est, Montréal, Québec, H1X 2B2, Canada

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Key Word Index—*Catharanthus roseus*; Apocynaceae; desacetoxyvindoline 4-hydroxylase; methyl jasmonate; salicylic acid; tryptophan decarboxylase; vindoline.

Abstract—Methyl jasmonate, a chemical inducer of secondary metabolism, has been shown to promote vindoline biosynthesis in developing seedlings, as a result of induction of tryptophan decarboxylase (TDC) and desacetylvindoline 4-hydroxylase (D4H). The present studies suggest that jasmonate-based induction of TDC and D4H activities involves modulation of transcriptional, post-transcriptional and post-translational controls. The effects of jasmonate on both enzymes were transient with maximum TDC activity appearing 12 h earlier than that of D4H. Jasmonate treatment of etiolated seedlings neither enhanced TDC activity nor could it replace the light requirement for D4H induction. Jasmonate, therefore, appears to modulate events which are already triggered by developmental and environmental specific controls. Salicylic acid, another chemical inducer of secondary metabolism, was ineffective in activating either TDC or D4H under the experimental conditions used.

INTRODUCTION

Numerous monoterpenoid indole alkaloids, including catharanthine and vindoline, are produced by the Madagascar periwinkle (*Catharanthus roseus*). As independent agents, both compounds have a limited ecological and therapeutic value [1, 2]. However, the oxidative coupling of these alkaloids produces the highly cytotoxic bisindole alkaloids vinblastine and vincristine. These dimeric alkaloids are currently used in cancer chemotherapy and they may represent important defensive/anti-feeding chemicals [2]. Unfortunately, both valuable anticancer agents are accumulated at very low levels in the aerial parts of *Catharanthus roseus* plants and intensive research has shown that this may be related to the strict controls which regulate the biosynthesis of the vindoline component of these dimers [3, 4]. Developing seedlings accumulate vindoline mostly in cotyledons where its biosynthesis is activated by a specific developmental program and by light [3, 5], whereas catharanthine accumulates throughout the seedling without light stimulation. Cell and organ cultures which accumulate catharanthine are unable to produce vindoline since they may lack certain cotyledon-specific enzyme activities involved in the late stages of vindoline biosynthesis [3, 5, 6]. However, a recent study has shown that transformation of cell cultures with different strains of *Agrobacterium tumefasciens* and *Agrobacterium rhizogenes* produced cell lines which express deacetylvindoline 4-*O*-acetyltransferase and accumulate low levels of vindoline [7].

Catharanthine and vindoline are derived from tryptamine and secologanin reviewed in [8]. Tryptophan decarboxylase (TDC; EC, 4.2.1.28) converts tryptophan into tryptamine and the iridoid glucoside secologanin is produced from geraniol by a series of enzyme reactions. The condensation of tryptamine and secologanin is catalyzed by the vacuolar enzyme strictosidine synthase (SS) to produce the key central intermediate strictosidine and several enzymatic reactions transform strictosidine by a series of different molecular rearrangements to form Iboga alkaloids, such as catharanthine, or Aspidospermane-type alkaloids, such as tabersonine and vindoline.

* Dedicated to Dr. Neil Towers on the occasion of his seventy-fifth birthday.

†Author to whom correspondence should be addressed: Tel: (514) 872-8492 Facsimile: (514) 872-9406 E-mail: delucavi@ere.umontreal.ca

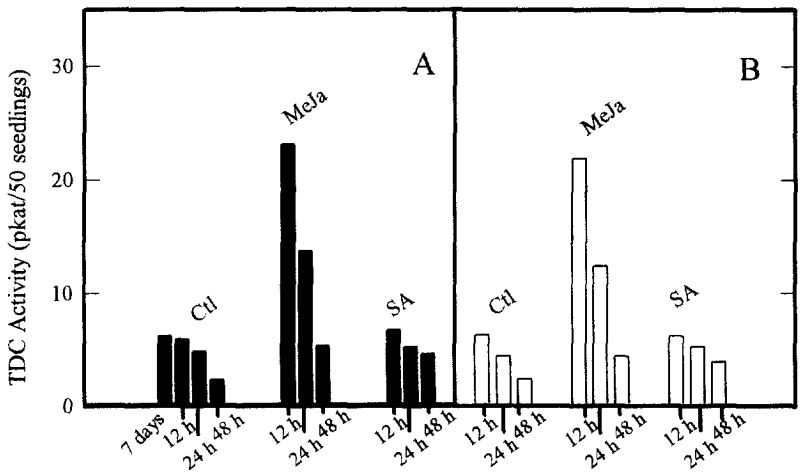

Fig. 1. *Effects of MeJa and SA on TDC activity during seedling development.* Seven-day old etiolated seedlings (7 D) were exposed to 1.25 ppm of MeJa vapor, 70 pmoles SA/seedling, or water (Control, Ctl). Seedlings were either kept in the dark (closed bars), or were exposed to light (open bars) for 12, 24 and 48 h, respectively. Each data point represents the average of two replicate experiments where the variation was less than 10% of the average.

The position of TDC at the interphase between primary and secondary metabolism suggests that it may play a critical role in controlling carbon flux towards alkaloid biosynthesis in *Catharanthus roseus* [3, 5, 9]. However, the appearance of maximum TDC activity in developing seedlings does not correspond with the time of vindoline accumulation which occurs 24 to 36 h after TDC activity reaches its peak. Accumulation of vindoline does, however, coincide with the appearance of maximal enzyme activities which catalyze the terminal steps of vindoline biosynthesis [3, 10], (Fig. 1). The second to last step in vindoline biosynthesis is catalyzed by a 2-oxoglutarate dependent dioxygenase known as desacetoxyvindoline 4-hydroxylase (D4H; EC. 1.14.11.11), [11, 12] which hydroxylates desacetoxyvindoline at position 4 to produce deacetylvindoline. The last reaction in this pathway involves the *O*-acetylation of deacetylvindoline which is catalyzed by acetyl CoA: deacetylvindoline 4-*O*-acetyltransferase (DAT) [3, 5]. The enzyme activities for D4H and DAT are almost exclusively detected in the cotyledons of light-exposed seedlings and in leaves of mature plants [3, 10–12], which coincides with the sites of vindoline accumulation. Recent studies reported that the methyl ester of jasmonic acid, methyl jasmonate, can augment the accumulation of vindoline in developing seedlings in part by increasing TDC, SS, D4H and DAT enzyme activities [13]. Jasmonates act as chemical messengers in the transduction of environmental signals which may activate defense mechanisms including secondary metabolism [14]. Salicylic acid has also been found to participate in this defense-related increase of secondary metabolism [15].

We are interested in the regulation of vindoline biosynthesis and in the external factors that affect it. In this article we report the effects of jasmonate and salicylic acid, two chemical inducers of secondary metabolism, on two key enzymes, TDC and D4H, involved in vindoline biosynthesis.

RESULTS AND DISCUSSION

Catharanthus roseus seedlings were germinated in darkness for 7 days and were then exposed to methyl jasmonate (1.25 ppm) or salicylic acid (70 pMoles/seedling) [21], under either a dark or a light regime. Light treatment was necessary to induce D4H activity [10, 12, 13] and vindoline accumulation, whereas the TDC activity profile was not altered significantly by light treatment [3, 5].

Effects of MeJa and SA on the development- and environment-specific regulation of TDC

The appearance of TDC activity has been shown to be under developmental and hormonal control [9, 16, 18, 19, 20, 21] and the enzyme peaks transiently for a brief 48 h period between day 4 to day 6 of seedling development [9, 19]. Jasmonate treatment of seven-day old seedlings which only contain 25% of the maximal activity found in 5-day old seedlings, resulted in a transient reactivation of TDC enzyme activity. A 12 h exposure of 7-day old seedlings to jasmonate vapors produced a transient 400% increase in TDC enzyme activity in both dark- (Fig. 1A) and light-grown (Fig. 1B) seedlings compared to untreated control seedlings, whereas TDC activity almost decreased to control levels after a further 36 hours of exposure to jasmonate. Previous studies showed that polyclonal anti-TDC antibodies reacted positively against several discrete proteins in crude seedling extracts which were resolved by SDS-PAGE and Western immunoblotting [9]. The slowest migrating proteins (72, 68 and 65 kD) were thought to represent ubiquitinated forms of TDC, whereas more rapidly migrating proteins were

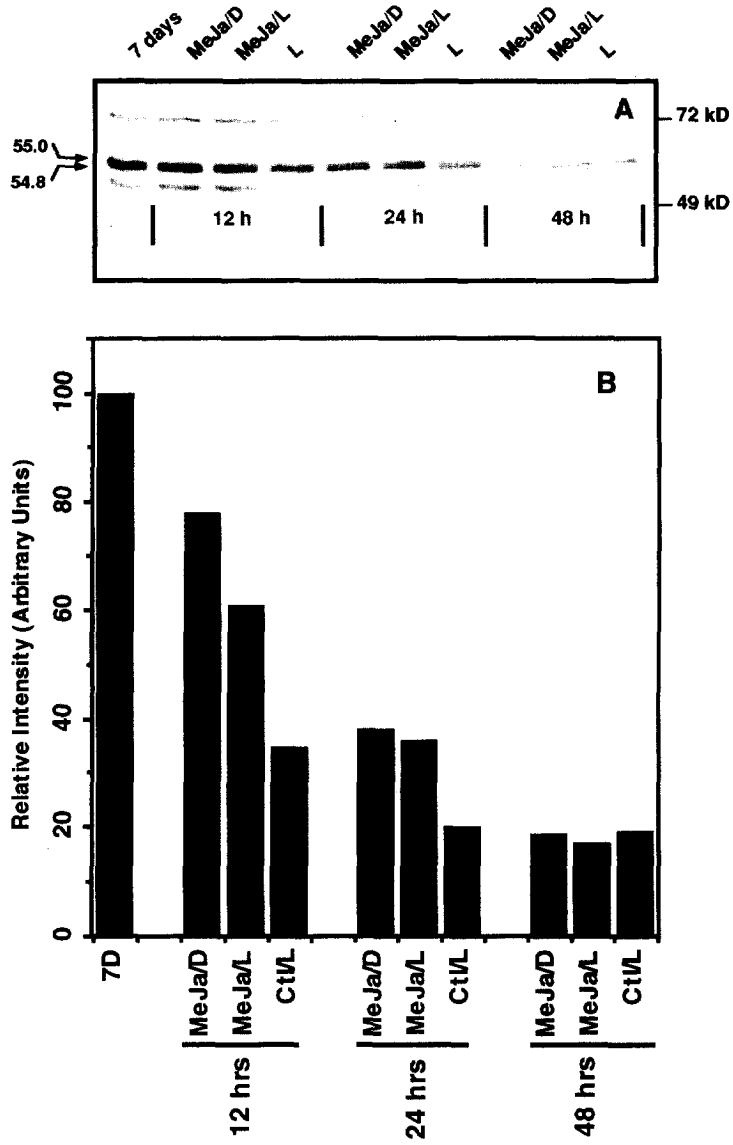

Fig. 2. *Effects of MeJa on TDC protein levels during seedling development.* Seven-day old etiolated seedlings (7 D) were either exposed to 1.25 ppm of MeJa vapor, or water (Control, Ctl) and were either kept in the dark (D), or were exposed to light (L) for 12, 24 and 48 h, respectively. Protein fractions equivalent to half a seedling were submitted to 12% SDS-PAGE, transferred onto a nitrocellulose membrane, and the membranes were probed with anti-TDC antibodies. The immunoblots were scanned by densitometry. The relative joint densities of the 54.6 and 55 kD immunoreactive proteins are displayed as a bar graph as a percentage of the most intense immunoblot.

proposed to be degradation products [17]. The appearance of TDC enzyme activity in developing seedlings coincided with the detection of a 54.8 and a 55 kD immunoreactive protein [9]. SDS-PAGE and immunoblotting of protein extracts from jasmonate-treated *Catharanthus roseus* seedlings, did not reveal any increase in the 55 kD immunoreactive protein over initial levels after a 12 h MeJa exposure (Fig. 2), but the levels of this immunoreactive protein decreased more slowly in jasmonate treated dark- or light-exposed seedlings than in the corresponding untreated light-control (Fig. 2). As TDC activity decreased after 24 and 48 h of jasmonate treatment, a corresponding reduction in both the 54.8 and 55 kD proteins was observed (Fig. 2) and these ultimately returned to the TDC antigen levels detected in untreated seedlings (not shown).

In its active form TDC appears to be a 110 kD homodimer [16, 18] composed of two 55 kD subunits. The equilibrium between the stable active dimer and an unstable monomer may involve post-translational modifications of the monomer which lead to inac-

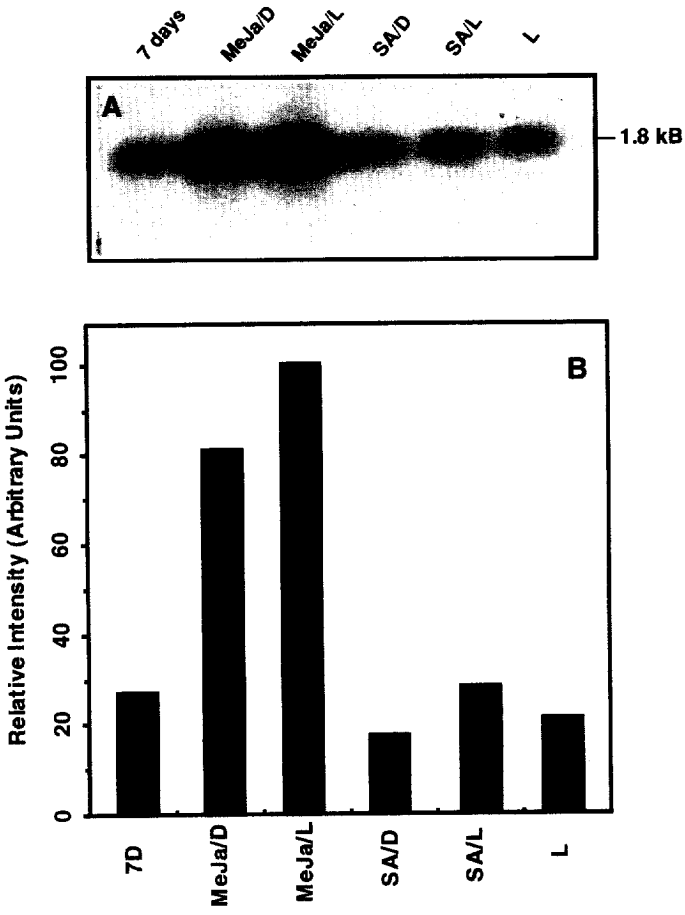

Fig. 3. *Effects of MeJa and SA on TDC transcript levels during seedling development.* Seven-day old etiolated seedlings (7 D) were either exposed to 1.25 ppm of MeJa vapor, 70 pmoles SA/seedling, or water (Control, Ctl) and were either kept in the dark (D), or were exposed to light (L) for 24 h. Ten μg of total RNA from each treatment were separated on formaldehyde containing agarose gels, transferred onto a nitrocellulose membrane, and probed with the cDNA clone TDC-5 radiolabelled with [^{32}P]. Northern blots were scanned by densitometry. The relative intensities of transcripts are expressed as a percentage of the most intense blot.

tivation and degradation of the enzyme [16]. In view of this proposed regulatory mechanism, a plausible interpretation of our results could be that jasmonate favors the maintenance of the stable active dimeric form of the enzyme (Fig. 2, 12 h treatment) compared to the situation in untreated control seedlings. Although jasmonate may stabilize active TDC protein, the transient induction of enzyme activity observed in Fig. 1 may also be due to increased transcription of TDC mRNA. In fact MeJa treatment for 24 h also caused a 3- to 4-fold increase of *tdc* transcript levels in dark- or light-grown seedlings (Fig. 3). These results corroborate previous findings which suggested that TDC is regulated by complex transcriptional, post-transcriptional and post-translational controls [9, 16, 17, 19, 20]. In contrast, salicylic acid did not promoting increases in either TDC activity (Fig. 1), antigen accumulation (data not shown) or TDC transcript levels (Fig. 3).

Effects of MeJa and SA on the development- and environment-specific regulation of D4H

The effects of jasmonate and salicylic acid on the expression of D4H during seedling development were also studied. Very low levels of enzyme activity were observed in 7-day old etiolated seedlings (Fig. 4), and neither jasmonate nor salicylic acid were effective in inducing D4H activity in the dark. Exposure of etiolated seedlings to light increased D4H enzyme activity [12] (Fig. 4, Ctl) and MeJa treatment could further transiently activate this enzyme. The increase of D4H enzyme activity was about 85% higher in seedlings exposed to jasmonate (Fig. 4B, MeJa at 24 h) than in controls (Fig. 54, Ctl. at 24 h). Longer exposures to MeJa resulted in a decrease of enzyme activity back to the corresponding illuminated control level (Fig. 4B).

It was noted previously (Vazquez-Flota, unpub-

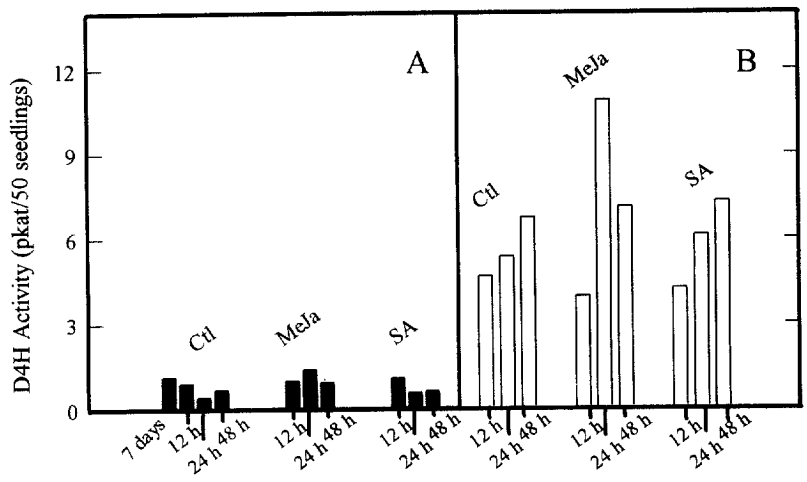

Fig. 4. *Effects of MeJa and SA on D4H enzyme activity during seedling development.* The treatments and symbols are described in Fig. 1. Each data point represents the average of two replicate experiments where the variation was less than 15% of the average.

lished) that depending on seedling development and the light regime, similar levels of D4H antigens could account for variations of up to 300% in enzyme activity. Under the conditions used in this experiment, significant levels of D4H protein (Fig. 5) were detected in the etiolated seedlings which have low levels of enzyme activity (Fig. 4A). Light-treatment for 48 h increased D4H activity up to 7-fold compared to dark controls (Fig. 4). In contrast, the level of D4H protein did not correlate with the enzyme activity as it decreased slightly over the dark control and subsequently surpassed slightly the dark control after 48 hours of light treatment. Recent studies in our laboratory which indicate that different isoforms of antigenic D4H protein exist in dark- and light-grown seedlings (Vazquez-Flota, unpublished) may explain the lack of correlation observed in Figs 4 and 5. Treatment of light-grown seedlings with MeJa resulted in shortening by 24 h the time necessary to detect maximal D4H protein (Fig. 5, MeJa/L at 24 h) and this correlated completely with the timing of the highest D4H activity (Fig. 4). In contrast to the effect observed with MeJa, salicylic acid treatment of light-grown seedlings did not transiently activate D4H enzyme activity (Fig. 4) or alter the pattern of accumulation of D4H protein (data not shown).

During the course of etiolated seedling development, *d4h* transcripts were shown to continuously increase to a maximum and then decreased to below detectable levels unless the seedlings were exposed to light [12]. Treatment of seven-day old etiolated seedlings with MeJa or SA in the absence of light did not affect the levels of *d4h* transcripts (Fig. 6). It was exclusively upon illumination that a significant 6-fold increase in transcript levels was detected (Fig. 6), but the simultaneous application of light and either jasmonate or salicylic acid did not further enhance transcript accumulation (Fig. 6). However, light- and MeJa-treatment increased D4H protein by 100% (Fig. 6, MeJa/L at 24 h) relative to control seedlings (Fig. 5, L at 24 h), whereas the steady-state levels of *d4h* transcript were similar in both cases (Fig. 6). Jasmonate treatment, therefore, also appears to stabilize or to extend the residence time of active D4H in some undetermined manner. For example, ribosomes isolated from jasmonate treated barley plants will preferentially translate defense-related transcripts over those of photosynthetic genes [22]. One can speculate that this or some other mechanism involving post-transcriptional or post-translational controls could also operate in *Catharanthus roseus*.

The results obtained in this report extend previous observations that MeJa treatment triggers measurable increases in indole alkaloid levels by modulation of both early and late stages of biosynthesis [13]. However, jasmonate appears only to modulate the events which are already triggered by development- and environment-specific controls. The timing of the MeJa triggered transient activation of TDC and D4H occurred over a 12 h gap, with the TDC peak of activity occurring first (Figs 1 and 4). Earlier studies with light-grown developing seedlings have shown that TDC maximum activity precedes the D4H activity peak by 24 to 36 h [3, 10]. The results presented here suggest that MeJa differentially regulates the cascade of events leading to the coordinate activation of these genes and that it may modulate some common regulatory control points which govern the kinetics of activation of the early and late stages of vindoline biosynthesis. Jasmonates are involved in several developmental processes [22], and while most of their favorable effects on secondary metabolism are associated with a transcriptional activation of genes [23–25], they can also exert their regulatory effects at the post-transcriptional and post-translational levels [22]. This appears to be the case for both TDC (Figs 2 and 3) and D4H (Fig. 5).

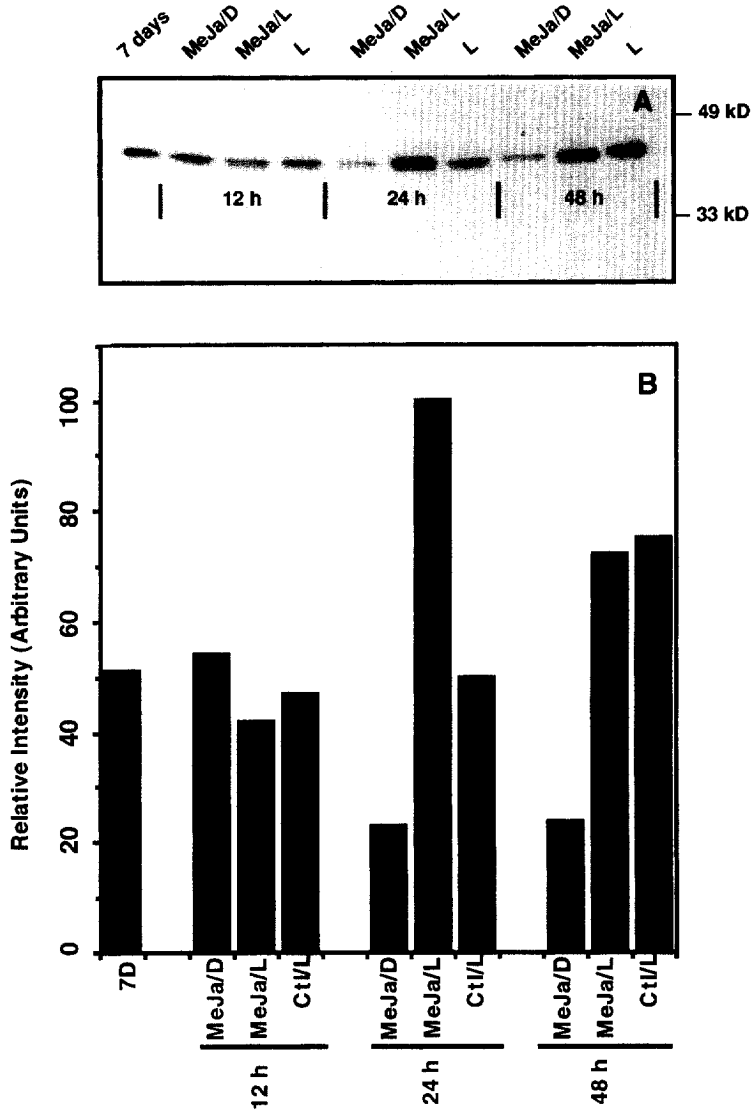

Fig. 5. *Effects of MeJa on D4H protein levels during seedling development.* The treatments and symbols are described in Fig. 2. The relative densities of the D4H immunoreactive protein are displayed as a bar graph as a percentage of the most intense immunoblot.

SA was ineffective in inducing any increase in TDC or D4H activities (Figs 1 and 4). Although it is possible that the applied concentration of SA was below the effective range, we adjusted it to the typical effective concentrations previously reported for other species [26]. Since SA is readily mobilized [26], we can assume that it was taken up and transported. In fact, hypocotyls of seedlings treated with salicylic acid were shorter and thicker than the controls, suggesting that the concentrations applied were in the effective range.

In conclusion, the results presented in this work stress the important roles played by development and by light to trigger vindoline biosynthesis in *Catharanthus roseus*. Other inducers of secondary metabolism such as MeJa appear to modulate events already initiated by seedling development and by light. Since MeJa was effective in altering TDC and D4H expression, this signaling molecule may be involved in transmitting development- or environment-triggered events that lead certain cells and tissues to produce alkaloids such as tabersonine or vindoline.

EXPERIMENTAL

Plant materials

Batches of *C. roseus* seeds (*cv.* Tropicana) were sterilized and planted on 3 layers of filter paper in 9 cm Petri dishes as described before [12]. Approximately 100 seeds were used per plate and care was taken to avoid contact between seeds. The plates were

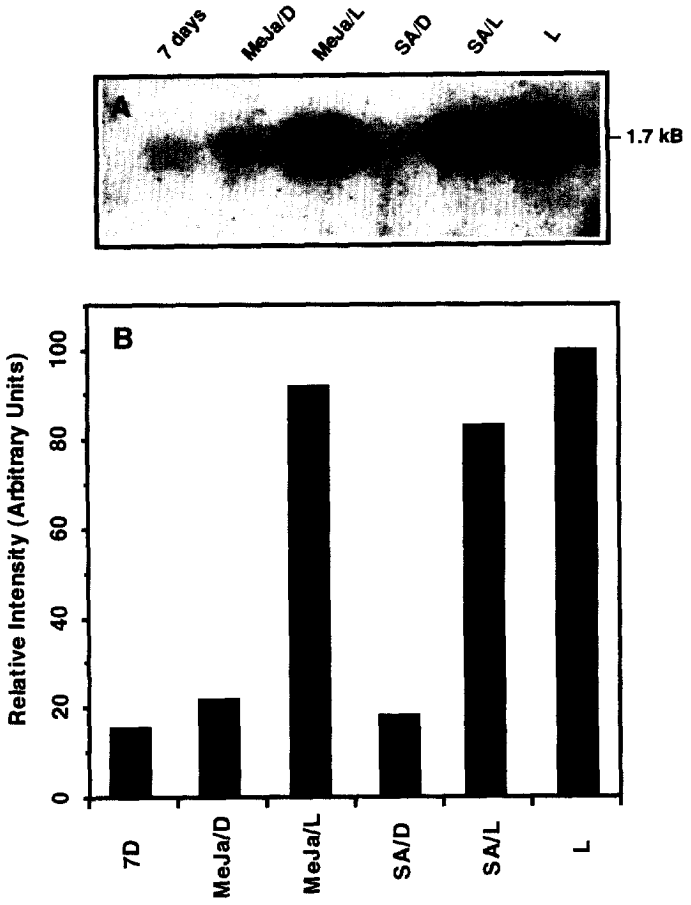

Fig. 6. *Effects of MeJa and SA on D4H transcript levels during seedling development.* The treatments and symbols are described in Fig. 2.

sealed with Parafilm and kept in the dark under controlled conditions for 7 days (25°C, 70% RH).

Treatments

Etiolated seedlings were exposed to MeJa or SA and were either kept in the dark or submitted to an 18 h photoperiod (20 μmol $m^{-2} s^{-1}$) for the periods shown in the Figures. MeJa (Firmenich) was diluted in ethanol and 25 μl of this solution was applied in a tiny cup which was placed in the center of the dish, avoiding any direct contact between the seedlings and the jasmonate solution. The final concentration of jasmonate in the internal atmosphere was adjusted to 1.25 ppm [13]. SA (BDH), was dissolved in 5% ethanol and 1 ml of the stock solution was applied underneath the paper layers to yield a final concentration of 7000 pmoles SA/petri dish. Samples were collected at the times indicated in the Figures, frozen in liquid nitrogen and kept at −80°C until analysis.

Enzyme analysis

Batches of 100 seedlings were ground in 2.5 ml of the extraction buffer containing 200 mM Tris-Cl pH 7.6, 10 mM EDTA, 5 mM dithiothreitol. After centrifugation, the supernatant was desalted on PD-10 columns (Pharmacia) previously equilibrated with 50 mM Tris-Cl pH 7.5 and 28 mM β-mercaptoethanol. TDC and D4H activities were assayed as described previously [3, 10].

Immunological analysis

Desalted extracts were mixed with SDS-loading buffer and the protein occurring in a half seedling (8 to 10 μg of protein) were applied and separated by SDS-PAGE [27]. After electrophoresis, proteins were transferred onto a nitrocellulose membrane for antigen detection [28]. TDC antigens were recognized with the anti-TDC antibody (H-95) [9], while D4H was detected with the anti-D4H antibody (D4H-ab) (Vazquez-Flota and De Luca, unpublished). Bound rabbit anti-TDC and -D4H antibodies were treated with donkey-anti-rabbit second antibodies coupled to horseradish peroxidase and products were detected by chemiluminescence (Renaissance kit from New England Nuclear).

Nucleic acid extraction and analysis

Total RNA was extracted, separated in agarose gels, and transferred to nitrocellulose membranes as described before [12]. *tdc* and *d4h* transcripts were detected by hybridization against the cDNA clones TDC-5 [29] and cD4H-3 [12], respectively, which had been radiolabelled with [^{32}P]. Conditions for hybridization and autoradiography are described in [12].

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