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Light activation of vindoline biosynthesis does not require cytomorphogenesis in *Catharanthus roseus* seedlings

Felipe A. Vázquez-Flota [1], Benoit St-Pierre [2], Vincenzo De Luca *

Institut de Recherche en Biologie Végétale, Département des Sciences Biologiques, Université de Montréal 4101 rue Sherbrooke est, Montréal (Québec), H1X 2B2, Canada

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This report is dedicated to Prof. Otto R. Gottlieb, on the occasion of his 80th birthday

Abstract

Upon illumination, the cotyledons of *Catharanthus roseus* seedlings readily synthesise vindoline from late biosynthetic intermediates, which accumulate in etiolated seedlings. The cellular localisation of tryptophan decarboxylase (TDC) and desacetoxyvindoline 4-hydroxylase (D4H), which catalyse the first and penultimate reactions of vindoline biosynthesis, was identified by immunocytochemistry in developing seedlings. The expression of TDC was restricted to the upper epidermis of cotyledons, whereas that of D4H was confined to laticifer cells. Light exposure of etiolated seedlings significantly induced D4H enzyme activity without changing the steady-state levels of D4H immunoreactive protein or modifying the cellular distribution of D4H expression in dark-grown seedlings. These results suggest that the early and late stages of vindoline biosynthesis occupy different cellular compartments, even in the early phases of etiolated seedling development. The role of light in activating the late stages of vindoline biosynthesis does not, therefore, seem to be related to the formation of the laticifer and idioblast cell types. It is concluded that light is not required for formation of these cell types, whereas regulatory factors, restricted to idioblasts and laticifers, may respond to light to activate localised expression of the late stages of vindoline biosynthesis.

Keywords: *Catharanthus roseus*; Apocynaceae; Development; Laticifer; Epidermis; Idioblast; Tryptophan decarboxylase; Deacetylvindoline-4-hydroxylase; Localisation

1. Introduction

The biosynthesis of vindoline in *Catharanthus roseus* (Apocynaceae) is a highly regulated process. During germination, vindoline intermediates of increasing complexity are accumulated as development proceeds (Scott, 1979; De Luca et al., 1986). The ontogenetic regulation of vindoline biosynthesis results from the coordinate expression of enzymes involved at the early and late stages of the process (De Luca et al., 1986, 1988). The biosynthesis of vindoline involves the formation of strictosidine, which is a common intermediate for the formation of the more than 100 monoterpenoid indole alkaloids of *Catharanthus* (Scott, 1979). Two reactions involved in strictosidine biosynthesis included tryptophan decarboxylase, which converts tryptophan into tryptamine and strictosidine synthase (STR1) which couples tryptamine with the iridoid-glycoside secologanin (Fig. 1). Different intramolecular rearrangements of the terpenoid moiety of strictosidine result in the formation of the corynanthe-, iboga and aspidosperma-type alkaloids (Fig. 1). Strictosidine can be converted into tabersonine by an essentially uncharacterised pathway (Scott, 1979) (Fig. 1) and tabersonine is converted into vindoline by a sequence of six enzymatic reactions (Balsevich et al., 1986; De Luca et al., 1986). The first four reactions transform tabersonine into desacetoxyvindoline, and the last two reactions involve 4-hydroxylation of desacetoxyvindoline to yield deacetylvindoline, followed by 4-*O*-

* Corresponding author. Present address: Novartis Agribusiness Biotechnology Research Inc., PO Box 12257, 3054 Cornwallis Road, Research Triangle Park, NC 27709-2257, USA Tel.: +1-919-541-8575; fax: +1-919-541-8585.

E-mail address: vince.deluca@nabri.novartis.com (V. De Luca).

[1] Present address: Unidad de Biología Experimental, Centro de Investigación Científica de Yucatán, Apdo. Postal 87 Cordemex 97310, Mérida Yucatán México.

[2] Present address: Laboratoire de Physiologie Végétale, U.F.R. des Sciences et Techniques, University of Tours, Parc de Grandmont 37200 Tours, France.

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Nomenclature

The numbering system used is as for aspidospermine alkaloids in Chemical Abstracts (Collective Substance Index V 106–115 12CS3 p 5731CS, 1987–1991.)

acetylation to yield vindoline (Fig. 1). These reactions are catalysed by desacetoxyvindoline 4-hydroxylase (D4H) and by deacetylvindoline 4-*O*-acetyltransferase (DAT), respectively (De Luca et al., 1988) (Fig. 1).

During seedling development, TDC and STR1 enzyme activities are expressed 36–48 h prior to those of D4H and DAT (De Luca et al., 1988). The activation of D4H and DAT coincides with the quantitative transformation of tabersonine into vindoline and requires the participation of light (Balsevich et al., 1986; De Luca et al., 1988). Light induction of D4H and DAT expression is apparently mediated by phytochrome, since a pulse of red light (660 nm) is enough to activate both enzymes and a subsequent exposure to far-red irradiation (710 nm) is enough to reverse these effects (Aerts and De Luca, 1992; Vázquez-Flota and De Luca, 1998).

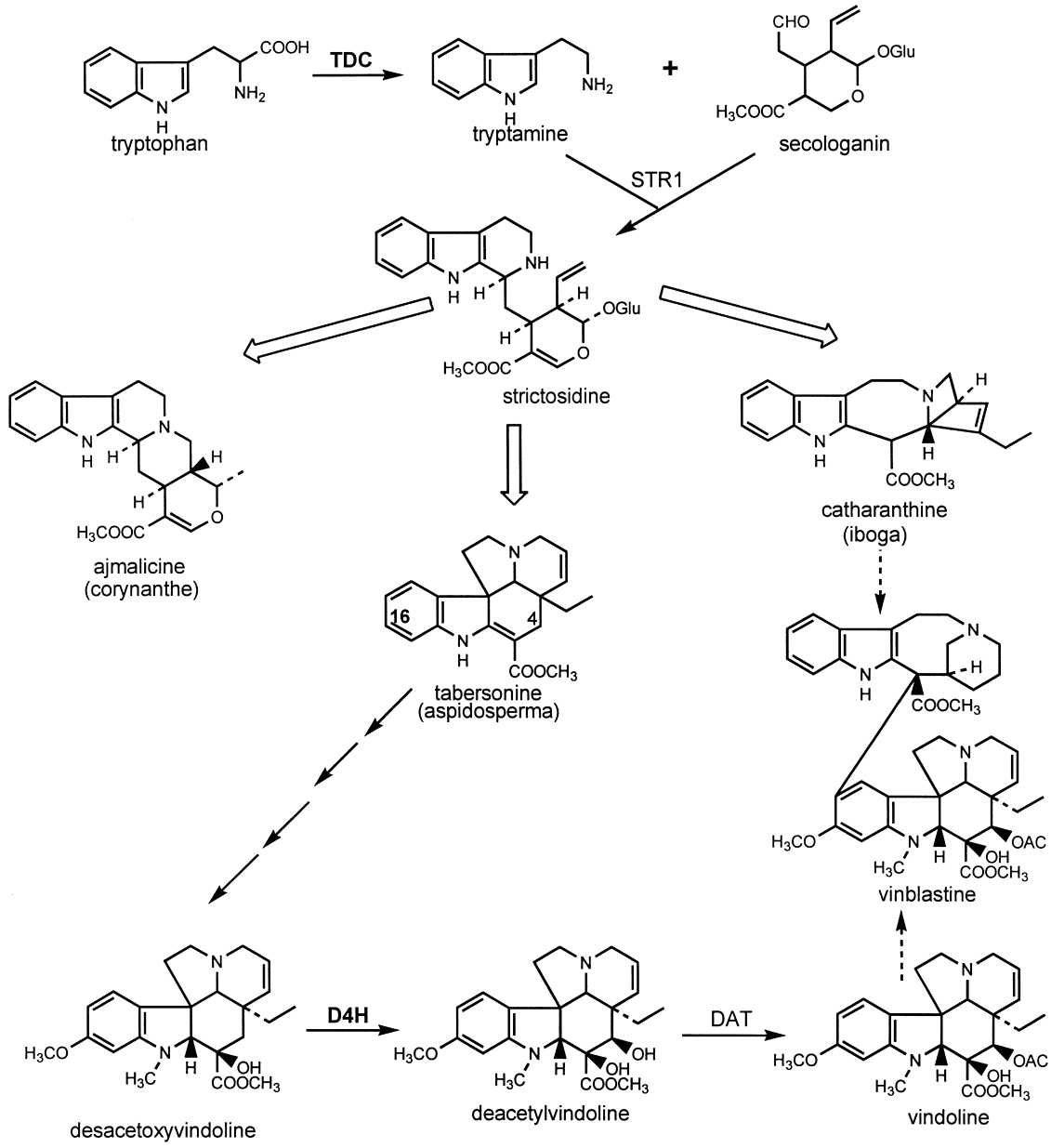

Fig. 1. Scheme of monoterpenoid indole alkaloid biosynthesis leading to the corynanthe, aspidosperma and iboga types, which are derived from the central intermediate strictosidine. Tabersonine (Aspidosperma) is converted to vindoline by 6 enzymatic steps. Vindoline is coupled enzymatically to catharanthine (iboga) to yield dimeric indole alkaloids like vinblastine. TDC, tryptophan decarboxylase; STR1, strictosidine synthase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline-4-*O*-acetyltransferase. Solid lines represent single reactions, dashed lines represent multiple reactions.

Recently, we reported the differential cell distribution of four enzymes involved in alkaloid biosynthesis in leaves of *Catharanthus* plants (St-Pierre et al., 1999). TDC and STR1 were found only in epidermal cells, whereas D4H and DAT were restricted to specialised cell types known as laticifers and idioblasts (St-Pierre et al., 1999). The differential cell distribution of these enzymes suggests a complex intercellular trafficking of strictosidine or a post-strictosidine intermediate that is required for vindoline biosynthesis within the specialised cell (St-Pierre et al., 1999). These results raised the possibility that light may also activate the development of laticifers and idioblast where these enzymes are localised. To address this possibility, we have applied an immunocytochemical approach to the localisation of TDC and D4H in tissues of developing seedlings.

2. Results and discussion

2.1. Tissue distribution of TDC and D4H

Seedlings of *Catharanthus roseus* were germinated for 6 days in the dark and then either exposed to light or kept in the dark for an extra 36-h period. The reproduction of previous studies (De Luca et al., 1986, 1988) show that cotyledons of 6 day old etiolated seedlings contained the highest levels of TDC enzyme activity regardless of the light regime used (data not shown). The TDC activity in hypocotyls and radicles was at most 30% of that observed in cotyledons, regardless of the stage of development and light regime used (Fig. 2A). Western immunoblots showed that the distribution of the 55 kDa active form of TDC (Alvárez-Fernández et al., 1989) followed a similar developmental pattern as that observed for the appearance of enzyme activity. However, immunoreactive forms with a higher M_R were also observed in all tissues (Fig. 2A, upper panel) and may represent ubiquitinated TDC polypeptides that target them to an ATP-mediated proteolytic process (Alvarez-Fernández et al., 1989; Alvarez-Fernández and De Luca, 1994).

D4H enzyme activity occurred at very low levels in dark-grown seedlings, whereas a 36-h light treatment produced a 400% increase in D4H activity that was restricted to cotyledons (Fig. 2B). Despite low levels of D4H activity in cotyledons of etiolated seedlings, immunoblots revealed the presence of a significant amount of D4H protein that was only slightly increased by light treatment [Fig. 2B, upper panel (Vázquez-Flota and De Luca, 1998)]. No D4H immunoreactive forms were detected in other tissues than cotyledons (Fig. 2B, upper panel).

2.2. Microscopic observation of Catharanthus cotyledons

Longitudinal sections from cotyledons were prepared from seedlings grown continuously in the dark for 7.5 days, or exposed to light for 36 h after 6 days of etiolated growth. Cotyledons from younger seedling were

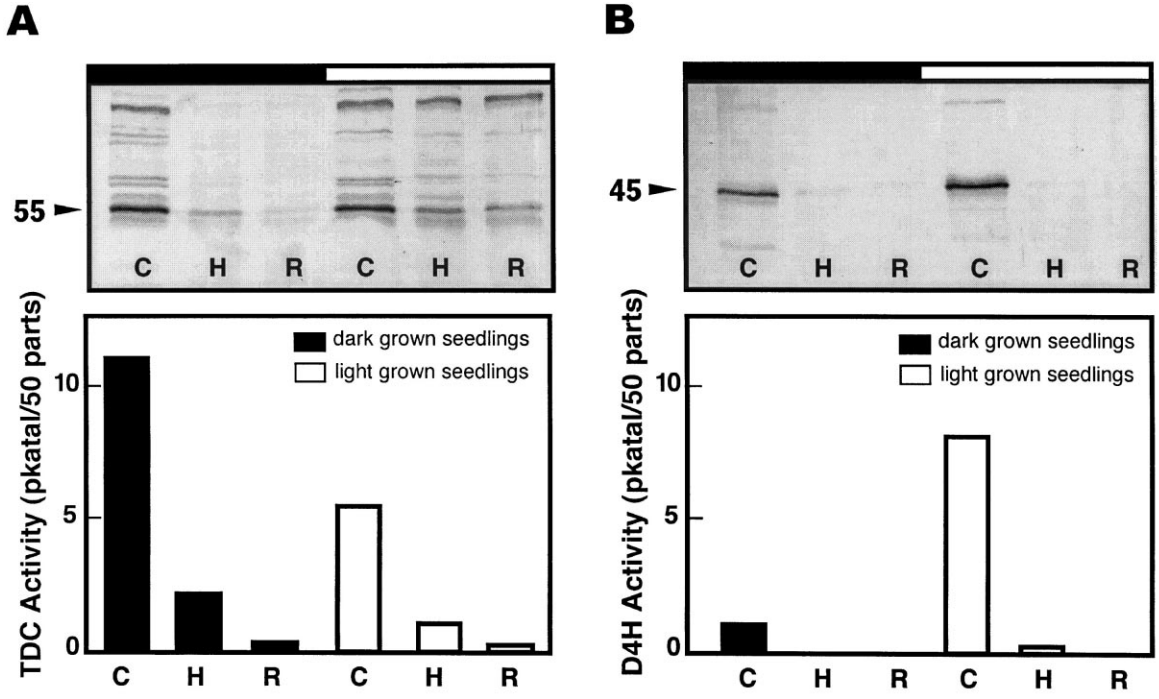

Fig. 2. TDC (A) and D4H (B) enzyme activities in 6 day old cotyledons (C), hypocotyls (H) and radicles (R) of dark (black bars) and light (open bars) grown *Catharanthus* seedlings. Upper panels show TDC and D4H immunoblots. The major immunoreactive froms of TDC and D4H had M_r of 55 and 45 kD, respectively. The antiserum dilutions were 1:2000 and 1:10000 fold for TDC and D4H, respectively. *Catharanthus* seedlings were grown for 7.5 days in continuous darkness or were exposed for 36 h to white light (20 μmol m^{-2} s^{-1}) after 6 days of dark growth.

too fragile and did not produce sections of quality sufficient for analysis. Sections from etiolated cotyledons (Fig. 3A and B) were anatomically similar to those of light exposed cotyledons (Fig. 3C and D) and to those from mature leaves (Yoder and Mahlberg, 1976; St-Pierre et al., 1999). A single layer of upper and lower epidermis cells surrounded the mesophyll, which consisted of a single layer of palisade parenchyma on top of several files of spongy parenchyma (Fig. 3). Laticifer cells, in the cotyledon vasculature, were observed at the interphase between palisade and spongy parenchyma (Fig. 3). In addition, longitudinally oriented, subdermal laticifers were identified as elongated structures scattered among the spongy mesophyll on the abaxial side of the cotyledon (Fig. 3). These laticifer cells had no apparent contact with the vascular vessels.

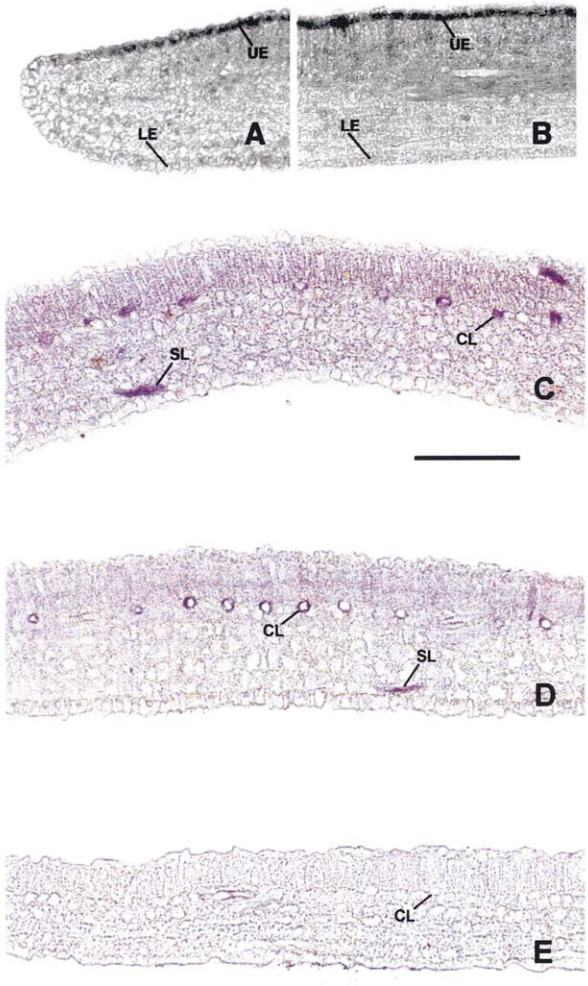

Fig. 3. Immunolocalisation of TDC and D4H proteins in cotyledons. Longitudinal sections of cotyledons from light-treated seedlings (A, C and E) and from etiolated seedlings (B, D) were used for immunolocalisation studies. (A) and (B) shows cotyledon sections after incubation with TDC antiserum (1:200). (C) and (D) shows cotyledon sections after incubation with D4H antiserum (1:500). (E) shows a cotyledon section incubated with pre-immune serum (1:200). CL, cross-connecting laticifer; LE, lower epidermis, SL, sub-dermal laticifer; UE, upper epidermis.

2.3. Immunocytochemical localisation of TDC and D4H

The anti-TDC antiserum recognised immunoreactive proteins in cells forming the upper epidermis of both light-treated (Fig. 3A) and dark-grown (Fig. 3B) cotyledons. Except for the lack of expression in lower epidermis, the localisation of TDC in the upper epidermis of cotyledons, is similar to that found in young leaves of mature plants (St-Pierre et al., 1999). The anti-D4H antiserum reacted with immunoreactive proteins within laticifer and idioblast cells that were distributed throughout the mesophyll (Fig. 3C and D). Furthermore, the anti-D4H antiserum recognised D4H immunoreactive protein within laticifers and idioblasts of both light-treated (Fig. 3C) and etiolated (Fig. 3D) seedlings. These results support our previous findings, which suggested that etiolated seedlings express D4H in an inactive form (Vázquez-Flota and De Luca, 1998). Light treatment appears to activate the D4H enzyme through an undetermined post-translational modification (Vázquez-Flota and De Luca, 1998), in addition to activating *d4h* gene expression. Control sections, probed with pre-immune serum, did not produce any visible immunoreaction (Fig. 3E).

The differential cellular distribution of TDC and D4H in cotyledons was similar to that found in leaves of mature plants (St-Pierre et al., 1999). In fact, in situ RNA hybridisation showed that STR1 expression was also restricted to epidermal cells in cotyledons (data not shown). These results corroborate that strictosidine biosynthesis occurs in the epidermis of *Catharanthus* leaves (St-Pierre et al., 1999) and cotyledons (Fig. 3A). The spatial separation of TDC (Fig. 3A and B) from D4H (Fig. 3C and D) suggests that at least two different cell types participate in vindoline biosynthesis in cotyledons of *Catharanthus* seedlings and in young leaves of mature plants, with the last steps occurring exclusively in specialised cells. Therefore, a potentially complex mechanism for the transport of strictosidine or post-strictosidine intermediates from epidermis or roots (St-Pierre et al., 1999) to laticifer cells may operate in *Catharanthus* tissues (St-Pierre et al., 1999). This study confirms that such a mechanism functions even in early phases of development within etiolated seedlings (Fig. 3) and that light appears not to be necessary for the development of laticifer or idioblast cells. No differences in shape or number of these specialised cells were observed between cotyledons from dark- and light-grown seedlings (Fig. 3C and D). In fact, laticifer cells were even shown to be present in mature embryos (Yoder and Mahlberg, 1976). The presence of cellular structures which accommodate vindoline biosynthesis within the earliest stages of etiolated seedling growth suggests that regulatory factors, which

are restricted to the idioblast and laticifer, may respond to light treatment by activating the late stages of vindoline biosynthesis (Fig. 3C and D). The presence of laticifer cells in cotyledons from dark-grown seedlings was confirmed by epifluorescence microscopy. The distinctive fluorescence of laticifers may be related to their different chemical composition (Mersey and Cutler, 1986; St-Pierre et al., 1999), that includes the presence of latex containing indole alkaloids.

The participation of laticifer cells in secondary metabolism has been well documented for several species (Fahn, 1988). Cell type-specific biosynthesis of biologically active compounds permits their sequestration from potentially sensitive cellular processes and their accumulation to high concentrations in specific and well defined cells. This process also involves subcellular differentiation, since *Catharanthus* cell cultures, grown under alkaloid producing conditions, have vacuoles with a different morphology from those cultured under normal conditions (Neumann et al., 1983). In cultured cells of *Berberis* and *Thalictrum*, the last two enzymes involved in the synthesis of berberine are located in a specialised vesicle (Amann et al., 1986). These observations suggest that the occurrence of subcellular structures may be a common feature of secondary metabolite producing cells. In fact, two functionally different vacuolar compartments have been identified in maize, pea and tobacco plants (París et al., 1996).

The differential distribution of enzymes involved in secondary metabolism is not exclusive for alkaloid biosynthesis. In parsley seedlings, the enzymes involved in general phenylpropanoid metabolism, such as phenylalanine ammonia lyase (PAL) and 4-coumarate: CoA ligase (4CL), were detected in several types of cells. However, the locations of chalcone synthase (CHS), which is committed to flavonoid biosynthesis, and *S*-adenosyl methionine: bergaptol *O*-methyltransferase (BMT), which is involved in furanocoumarin biosynthesis, were mutually exclusive. Expression of CHS was restricted to the epidermis, spongy mesophyll and cells surrounding the oil duct, whereas BMT only occurred in the vascular bundle, palisade parenchyma and the oil duct epithelial cells (Jahnen and Hahlbrock, 1988; Reinold and Hahlbrock, 1997).

The formation of cytotoxic dimeric alkaloids from the oxidative coupling of catharanthine and vindoline occurs later in plant development than the period involved in formation of either alkaloid (Aerts et al., 1996; Naaranlahti et al., 1991). The precise site of catharanthine synthesis and dimer formation remains unknown, but spatial separation of these components could provide an extra regulatory point of control for dimer formation. The compartmentation of dimer formation within specialised cells may represent a mechanism for producing cytotoxic dimeric alkaloid, without killing the plant host.

3. Experimental

3.1. Plant materials

Seeds of *Catharanthus roseus* cv. Little Delicata were germinated in the dark and subsequently exposed to light as described (De Luca et al., 1986). After harvest, seedlings were dissected into cotyledons, hypocotyls and radicles and either frozen in liquid nitrogen, or fixed for microscopy.

3.2. Analytical techniques

Tissues were extracted and analysed for TDC and D4H enzyme activities according to De Luca et al. (1986) and De Carolis et al. (1990), respectively. Procedures for SDS–PAGE and immunoblot have been described before (Vázquez-Flota and De Luca, 1998). Polyclonal antibodies against TDC and D4H were raised in New Zealand rabbits (Alvarez-Fernández et al., 1989; Vaśzquez-Flota and De Luca, 1998).

3.3. Immunocytochemical localisation of TDC and D4H

Cotyledons from dark- and light-grown *Catharanthus* seedlings were fixed and prepared for microscopy (St-Pierre et al., 1999). In situ immunolocalisation of TDC and D4H were performed as described in (St-Pierre et al., 1999). Briefly, sections mounted in slides were blocked with 3% BSA overnight at 4°C and then incubated in the primary antiserum for 2–4 h at room temperature. Unbound antibodies were washed with a saline solution buffered with Tris and containing Tween (TBST). Afterwards, the sections were incubated for 2 h with the secondary antibody coupled to alkaline phosphatase (BioRad, Hercules, CA). Unreacted secondary antibodies were removed by subsequent washes with TBST and an alkaline carbonate buffer (pH 9.0). A standard NBT/BCIP chromogenic reaction was used (St-Pierre et al., 1999).

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