TISSUE DISTRIBUTION OF TRANSCRIPTS INVOLVED IN BIOSYNTHESIS OF BENZYLISOQUINOLINE ALKALOID IN MATURE PLANTS OF *Argemone mexicana* L. (Papaveraceae)

DISTRIBUCIÓN TISULAR DE TRANSCRITOS INVOLUCRADOS EN LA BIOSÍNTESIS DE ALCALOIDES BENCILISOQUINOLÍNICOS EN PLANTAS MADURAS DE *Argemone mexicana* L. (Papaveraceae)

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SUMMARY

The distribution of berberine and sanguinarine was analyzed in roots, stems and leaves of mature *Argemone mexicana* plants, along with that of transcripts corresponding to selected genes involved in both early biosynthetic reactions, which are common to both alkaloids, and in the late specific reactions conducting to the formation of each of them. Roots were the main sites of alkaloid accumulation, though they showed the lowest accumulation of the analyzed transcripts. Results are discussed in terms of the operation of a possible transport mechanism of alkaloids between the aerial tissues and the roots, or the occurrence of different biosynthetic alternative reactions in both parts, aerial and underground tissues, involving different gene products, yet with similar catalytic capacities.

Index words: *Argemone mexicana*, benzylisoquinoline alkaloids, berberine, sanguinarine.

RESUMEN

La distribución de berberina y sanguinarina se analizó en raíces, tallos y hojas de plantas adultas de *Argemone mexicana*, junto con la de los transcritos correspondientes a genes seleccionados involucrados tanto en las reacciones biosintéticas iniciales, que son comunes para ambos alcaloides, como en las reacciones específicas finales que conducen a la formación de cada uno de ellos. Las raíces fueron los sitios de mayor acumulación de alcaloides a pesar de que mostraron la menor acumulación de los transcritos analizados. Estos resultados se discuten en función de la operación de un posible mecanismo de transporte de alcaloides entre los tejidos aéreos y las raíces o bien, de la ocurrencia de alternativas biosintéticas diferentes en tejidos de ambas partes, aéreos y subterráneos, con productos génicos diferentes pero con similares capacidades catalíticas.

Palabras clave: *Argemone mexicana*, alcaloides bencilisoquinolínicos, berberina, sanguinarina.

INTRODUCTION

The genus *Argemone* (Papaveraceae) comprises more than 25 species. Its center of distribution includes Southwestern USA and Northern Mexico (Schwarzbach and Kadereit, 1999). Several of those species are part of the native Mexican flora, but *A. mexicana* is the most successful and widely spread one. This plant, commonly known as chicalote or cardo in Mexico and as Mexican prickly poppy in the US, is considered a persistent and difficult to eradicate weed (Villaseñor y Espinosa, 1998). Nevertheless, it is also regarded as a medicinal plant, used since prior the arrival of Europeans to America (Rubio-Piña and Vázquez-Flota, 2013).

Furthermore, the plant has industrial application in the elaboration of environmentally-friendly agrochemicals (Hernández-Romero *et al.*, 2005). The bioactivity of this plant is attributed to the presence of benzylisoquinoline alkaloids (BIA's), which are distributed through the entire plant (Brahmachari *et al.*, 2013). More than 30 BIA's have been identified in *A. mexicana*; the most abundant ones correspond to sanguinarine and berberine, belonging to the benzophenanthridine- and protoberberine-types, respectively (Figure 1; Rubio-Piña and Vázquez-Flota, 2013). These alkaloids are distributed by following tissue-related patterns in mature plants. Sanguinarine is restricted to the roots and mature seeds, whereas berberine is found in both roots and aerial tissues (leaves and stems) (Rubio-Piña and Vázquez-Flota, 2013). Remarkably, the alkaloid distribution in this species changes according to the developmental stages. Sanguinarine is not found in mature plants but it is detected in leaves of young plantlets instead, while berberine is absent in the underground tissues of developing seedlings (Xool-Tamayo *et al.*, 2017a).

In developing seedlings and young plantlets the sites of alkaloid accumulation differ from those displaying the transcriptional activity related to its synthesis, suggesting the operation of complex regulatory mechanisms, which

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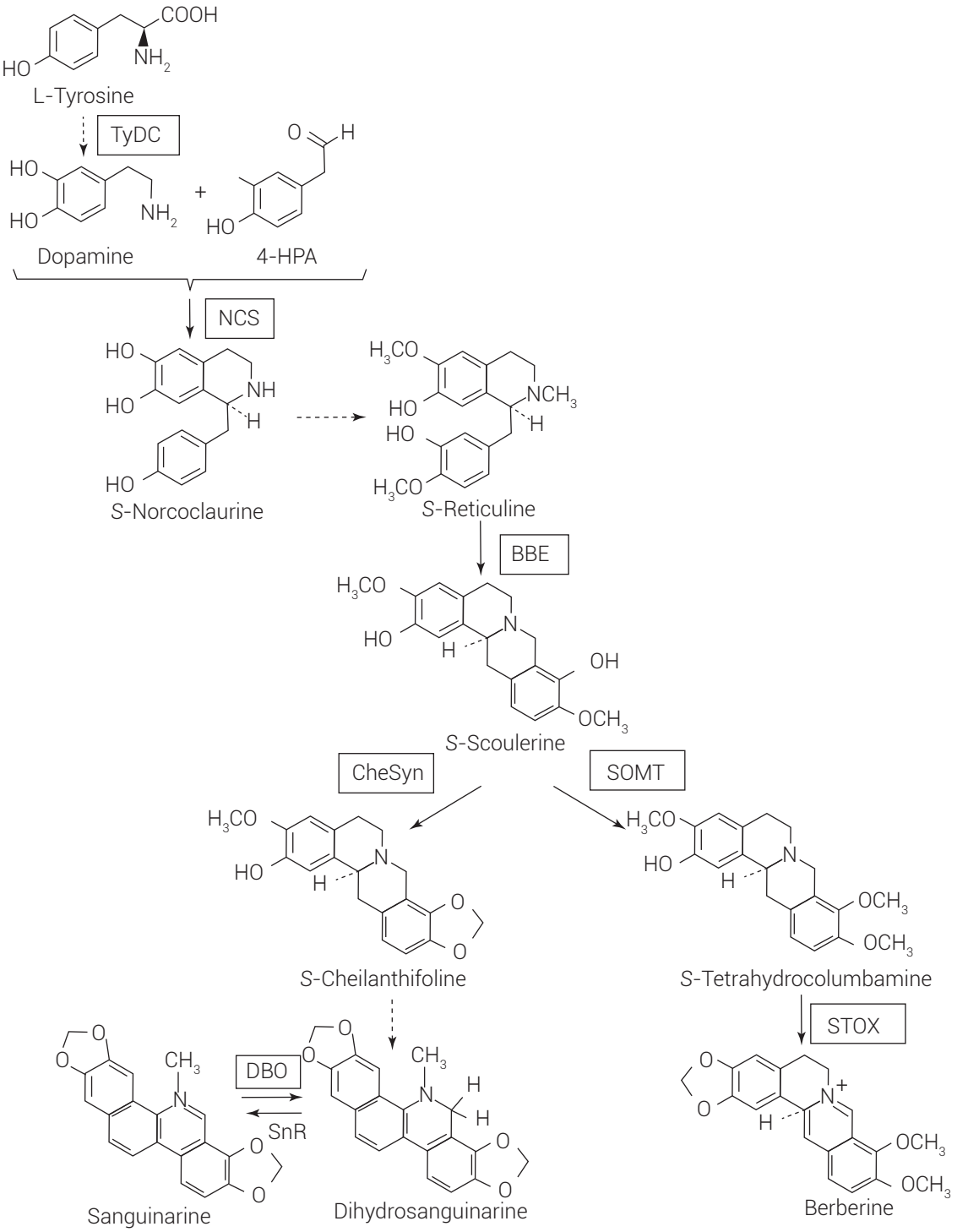


Figure 1. A condensed view of the biosynthetic pathway of benzylisoquinoline alkaloids. 4-HPA: 4-hydroxyphenylacetaldehyde; TyDC: tyrosine decarboxylase; NCS: norcoclaurine synthase; BBE: berberine bridge enzyme; SOMT: S-scoulerine O-methyltransferase; STOX: tetrahydroprotoberberine oxidase; CheSyn: cheilanthifoline synthase; DBO: dihydrobenzophenanthridine oxidase; SnR: sanguinarine reductase. Boxed enzymes correspond to those analyzed in this study, dashed arrows show multiple reactions.

could involve posttranscriptional controls, long distance transport or enzyme catalytic flexibility (Xool-Tamayo *et al.*, 2017a, b). This aspect has not yet been explored in mature plants which simultaneously could accumulate higher amounts of both berberine and sanguinarine than developing tissues. Since the formation of these alkaloids shares a common set of early biosynthetic intermediaries, the identification of the actual sites involved in the process could reveal the mechanisms sorting them among the final products.

Several members of the Papaveraceae family produce sanguinarine and, in all cases, both synthesis and accumulation take place in roots (Hagel and Facchini, 2013). In contrast, berberine, which rarely occurs in Papaveraceae but commonly occurs in Berberidaceae and Ranunculaceae, can either remain into the same tissue where it is formed, as it happens in *Thalictrum flavum* (Samanani *et al.*, 2005), or be exported to distantly located accumulation sites, as in *Coptis japonica* (Shitan *et al.*, 2003). BIA synthesis requires two units of tyrosine, one of them is deaminated by the action of aromatic monoamine oxidase (MAO), while the other one is hydroxylated and decarboxylated, involving the participation of tyrosine decarboxylase (TyDC). The resulting products, 4-hydrophenylacetaldehyde (4-HPA) and L-3, 4-dihydroxyphenylalanine respectively, are condensed forming norcoclaurine by the action of norcoclaurine synthase (NCS), which after two *O*- and one *N*-methylations is converted into reticuline (Figure 1).

The formation of an intramolecular carbon-carbon bond between the *N*-methyl group and the phenolic ring of reticuline, catalyzed by the berberine bridge enzyme (BBE), produces *S*-scoulerine which is the last common intermediary for the synthesis of both berberine and sanguinarine (Figure 1). This compound is used either by the *S*-scoulerine *O*-methyltransferase (SOMT) for berberine synthesis, or by the *S*-cheilanthifoline synthase (CheSyn) for sanguinarine synthesis (Hagel and Facchini, 2013). The formation of a 2,3 methylenedioxy bridge (O-CH_2-O), catalyzed by the tetrahydroprotoberberine oxidase (STOX) is the last step in berberine biosynthesis (Figure 1), whereas the formation of an additional 9,10 methylenedioxy bridge and further oxidation are required for sanguinarine synthesis (Figure 1).

Most biosynthetic genes have been isolated in different species and the corresponding orthologous genes have been identified in transcriptomic collections from *A. mexicana* stems, cell cultures and developing seedlings (Farrow *et al.*, 2012; Gesell *et al.*, 2011; Hagel *et al.*, 2015; Xiao *et al.*, 2013; Xool-Tamayo *et al.*, 2017a). Moreover, in some cases heterologous expression has confirmed identity and functionality (Díaz *et al.*, 2011). The aim of this study was to set an approach to establish the possible site of alkaloid formation in mature plants. We followed the transcript accumulation of genes participating at different stages of the biosynthetic process and compared them to those of the main alkaloids, sanguinarine and berberine. This analysis included genes involved in the common reactions required for the synthesis of both alkaloids, as well as those participating specifically in one or another branch (Figure 1).

MATERIALS AND METHODS

Plant material

Mature plants of *A. mexicana* displaying flowers and seed capsules were collected at the outskirts of Mérida, Mexico (20º 58' N, 89º 37' W) and taken to the lab where they were washed and dissected into leaves, stems and roots. Tissue samples were used for both alkaloid and nucleic acid analysis. Alkaloid analysis was performed on tissues collected at three different times, April 2012, May 2014 and April 2015, whereas transcripts were analyzed on plants from the last two collections.

Alkaloid extraction and quantitation

Tissues were freeze-dried, ground to a fine powder and used for alkaloid analysis. Alkaloids were extracted from 200 mg powdered tissues in 10 mL 0.5 % hydrochloric acid in methanol. For alkaloid identification, extracts were chromatographically separated on a Hypersil Gold™ C18 column (100 × 4.6 mm × 5 µm, Agilent Technologies) using a gradient of 95 % 10 mM ammonium acetate in acetonitrile pH 5.5 (A) in acetonitrile (B) created by a binary pump Agilent Series 1100 series controlled by the ChemStation 03.02 package. Chromatographic run started at 100 % A, reducing to 50 % in B (v/v) during the first 10 min, and then to 1 % in the up following 2 min, keeping it up for 1 min before returning to the initial condition, up to end of the analysis (total time 17.5 min).

Alkaloids were identified based on their positive-ion electrospray ionization (ESI) mass spectra obtained using a Bruker Apex III ion resonance mass spectrophotometer (Bruker Daltonics Inc., Billerica, MA, USA) and comparing with reported values (Liscombe *et al.*, 2009). Berberine, chelerythrine and sanguinarine were chromatographically quantified as described by Guízar-González *et al.* (2012) and Monforte-González *et al.* (2012).

Nucleic acid analysis

Total RNA was extracted from the same tissues used for alkaloid analysis (Rubio-Piña and Vázquez-Flota, 2008). Primers for *TyDC* (Acc. EU881888), *NCS-1* and *-2* (Acc.

EU881891 and EU881893), *BBE-1* and *-2* (Acc. EU881889 and EU881890), *SOMT* (Acc. KT984756), *STOX* (HQ116698) and *CheSyn* (*CYP719A14*; EF451152) were designed based on *A. mexicana* sequences available (Gesell *et al.*, 2011; Xiao *et al.*, 2013) and using the primer BLAST software (Table 1). Sequence functional identities had previously been assigned after bioinformatic analysis (Xool-Tamayo *et al.*, 2017a). A putative *AmDBOX* (UN11221) was retrieved from the seedling transcriptome (Xool-Tamayo *et al.*, 2017a) using as query *Papaver somniferum FADOX5* (Acc. UN108662; Hagel *et al.*, 2012).

For real time quantitative PCR (qPCR), first strand cDNA was synthetized using the M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer instructions and 500 µg μL^{-1} of oligo(dT) primer AP in a 20 µL reaction. The produced cDNA was diluted and stored at -80 ºC until analysis. Reactions were performed in a final 20 µL volume, containing 10 µL of the SYBR® Green Master Mix (Applied Biosystems, UK), 10 µM of each primer and 5 ng of the RT products. PCRs were performed using Eco™ Real Time PCR System (Illumina, San Diego, CA, USA) following a program of 35 cycles of 50 s at 95 ºC for DNA denaturation and 40 s at 60 ºC for primer alignment and amplification (Xool-Tamayo *et al.*, 2017a). Previously, the reaction mix was heated during 3 min at 95 ºC for enzyme activation. The melting curve was one cycle at 95 to 55 ºC and 55 to 95 ºC for 15 s (the fluorescence data was collected every 0.3 ºC during melting). The cycle threshold value (Ct) for each PCR was calculated using EcoStudy software (Illumina, San Diego, CA, USA). Transcript relative abundance was quantified by the Ct method, using actin as a reference (Simon, 2003).

RESULTS

Based on their chromatographic characteristics and mass fragmentation patterns, main alkaloids in *A. mexicana* corresponded to berberine (*Rt* 8.20 min; 336 $[M+H]^+$ *m/z*), sanguinarine (*Rt* 9.17 min; 332 $[M+H]^+$ *m/z*), dihydrosanguinarine (*Rt* 6.12 min; 334 $[M+H]^+$ *m/z*), and chelerythrine (*Rt* 9.00 min; 348 $[M+H]^+$ *m/z*). Combined, these alkaloids accounted for over 70 % of the total content. In addition, allocryptopine, protopine, canadine, columbanine and stylopine were also identified (Table 2). Alkaloids chelyrubine, N-methyl-stylopine and the biosynthetic intermediaries reticuline, methyl-coclaurine and scoulerine were also identified as minor components in the extracts.

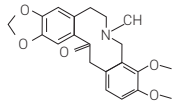
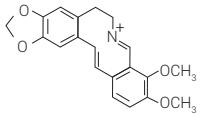
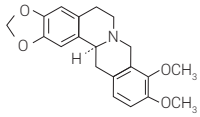
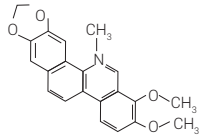
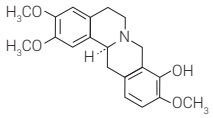
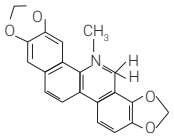
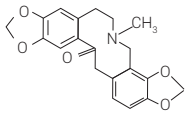
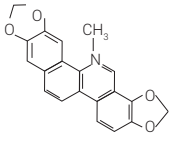
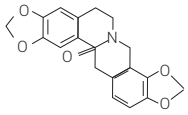
A tissue-associated distribution was detected for the

Table 1. Sequence of the primers used for PCR amplification (Xool-Tamayo *et al.*, 2017 a, b). See Figure 1 for abbreviations.

Label	Sequence (5′ to 3′)	Direction
Actin	CACIACTACTGCTAAACGGGAAA	F
	ACATCTGCTGGAAGGTGCTG	R
TyDC	GTTGAACCAGGTTATTTACG	F
	CGTATTCTTTCGCAACCTC	R
NCS1	CATCGCTAATTACGTTCTCAAGAATCA	F
	ATAGTAGTACATGGAATTACCTGGATGGGA	R
NCS2	CGTACCATTGAAATCCATGTCAGAA	F
	CATCGGACGGTAATTACCCATG	R
BBE1	CATCTTTGTTCATCATCATCTTCTTCTTCTTCTT	F
	GATCCTCTTGTGCAACATCTAACGGT	R
BBE2	CTCATCTTTGTTCATCTTCTTTTCTGTGC	F
	GATCCTCTTGTGCAACATCTAACGGT	R
CheSyn (CYP719A14)	AGGTCTTCAAGGTGTTGCCC	F
	TCTTTTCCCGCCCGTAACAT	R
SOMT	ATCCTATCCATGTCTACGAGGGCTATT	F
	CCAGTACCACCACCAACATCTAACA	R
STOX	GGTTAGGAAATATGGACTTG	F
	ATAACATTGCTGGTGAATCT	R
DBO	CACAGGCCAAGAGGTTTGCTA	F
	GCTTCCTCCTATTTTCTTTCCCTTT	R

F: forward; R: reverse.

Table 2. Alkaloids detected in the extracts from roots (R), stems (S) and leaves (L) from *A. mexicana*. Alkaloids were identified according their chromatographic retention time (*Rt*) and positive-ion ESI mass spectra.

Alkaloid and type	Structure	Distribution	*Rt* (min)	$[M + H]^+$ *m/z* (relative intensity)
Allocryptopine Protopine	 $C_{21}H_{24}NO_5^+$	R	6.92	370.16 (50.83)
Berberine Protoberberine	 $C_{20}H_{18}NO_4^+$	R, S, L	8.20	336.12 (66.56)
Canadine Protoberberine	 $C_{20}H_{21}NO_4^+$	R, S, L	5.27	339.14 (43.86)
Chelerythrine Benzophenathridine	 $C_{20}H_{18}NO_4^+$	R	9.00	348.20 (54.43)
Columbanine Protoberberine	 $C_{20}H_{20}NO_4^+$	R, S, L	7.61	338.13 (11.48)
Dihydrosanguinarine Benzophenathridine	 $C_{20}H_{20}NO_4^+$	R	6.12	334.10 (12.87)
Protopine Protopine	 $C_{20}H_{20}NO_5^+$	R	6.45	354.13 (98.83)
Sanguinarine Benzophenathridine	 $C_{20}H_{11}NO_4^+$	R	9.17	332.00 (86.01)
Stylopine Benzophenathridine	 $C_{19}H_{17}NO_4^+$	R	10.92	324.00 (24.82)

aforementioned alkaloids since benzophenanthridines (dihydrosanguinarine, chelerythrine, sanguinarine and stylopine), and protopines (allocryptopine and protopine), mainly occurred in roots, whereas protoberberines (berberine, canadine and columbamine) occurred in both aerial and underground parts (Table 2). Major alkaloids were quantified in leaves, stems and roots (Figure 2). Data shown correspond to the last plant collection, although similar results were obtained in all collected batches. Roots accumulated similar amounts of both berberine and sanguinarine (around 1.2 mg g DW^{-1}), with lower amounts of chelerythrine. In aerial parts, only berberine was detected, where it was present in higher amounts than in roots (2.0 mg g DW^{-1}) (Figure 2). Berberine in roots represented around 10 % in relation to the sanguinarine content, whereas chelerythrine, which is formed from dihydrosanguinarine (Figure 1), represented less than 5 % (Figure 2). In this way, roots from mature plants proved capable of accumulating alkaloids from the benzophenanthridine, protopine and protoberberine groups whereas aerial tissues only seem to have the capacity to accumulate protoberberine (Figure 2).

In order to assess the biosynthetic capacity of the *A. mexicana* tissues, the abundance of selected transcripts was estimated by qRT-PCR (Figure 3). Transcripts corresponding to genes involved in the early, common biosynthetic reactions (TyDC, NCS and BBE) were selected along with those participating specifically in either berberine (SOMT and STOX), or sanguinarine (CheSyn) synthesis (Figure 1). All the selected genes were expressed in the analyzed tissues; however, important differences in transcript relative abundances were noticed (Figure 3). Interestingly, even when roots showed the highest total alkaloid content (per dry matter unit; Figure 2), low transcript accumulation was observed for both genes involved in the early common biosynthetic reactions (TyDC, NCS and BBE) and likewise for those participating only in berberine synthesis (SOMT and STOX) (Figure 3). In contrast, *CheSyn* transcripts accumulated majorly in roots in comparison to the other organs (Figure 3). Stems and leaves showed similar levels of transcript accumulation in all cases, with the only exception for those corresponding to *CheSyn*, which were barely detectable in leaves, but accumulated at fair amounts in stems (Figure 3). It is interesting to notice that the two NCS and BBE isoforms were equally expressed in the analyzed tissues (Figure 3). In contrast to aerial tissues, roots displayed low gene expression associated to berberine synthesis (Figure 3).

The accumulation of *CheSyn* transcripts in stems is noteworthy (Figure 3) since no sanguinarine (nor other of the benzophenanthridine type alkaloids) was detected in aerial tissues (Figure 2). To further analyze the sanguinarine biosynthetic branch, DBO transcript accumulation was also included in this study (Figure 3). The putative *AmDBO* was previously retrieved from the *A. mexicana* seedling transcriptome (Xool-Tamayo *et al.*, 2017b). Interestingly, the amounts of *AmDBO* transcript were similar in stems and roots (Figure 3), regardless the differences of sanguinarine content (Figure 2), supporting the participation of stems in the synthesis of this alkaloid.

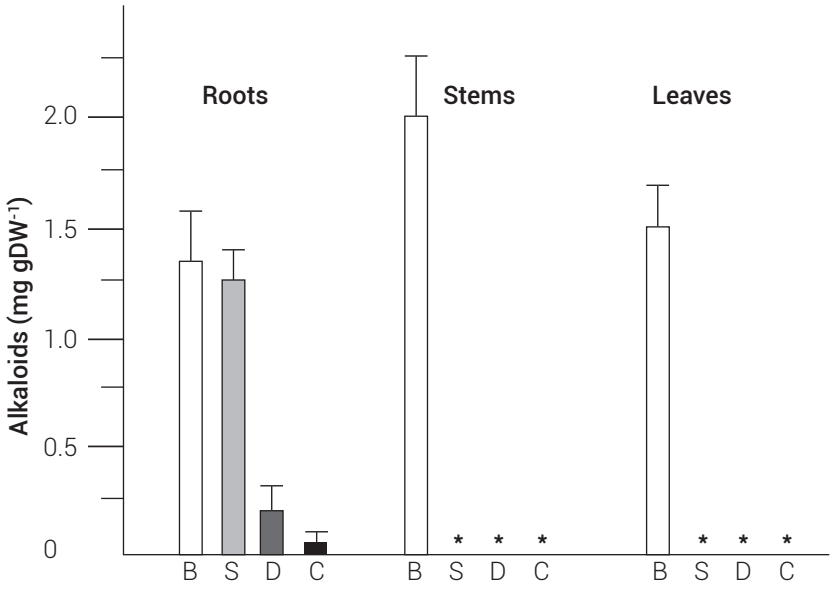


Figure 2. Alkaloid contents in roots, stems and leaves of *A. mexicana* plants. B: berberine; S: sanguinarine; D: dihydrosanguinarine; C: chelerythrine; ∗: Undetected. Average of three replications with standard error.

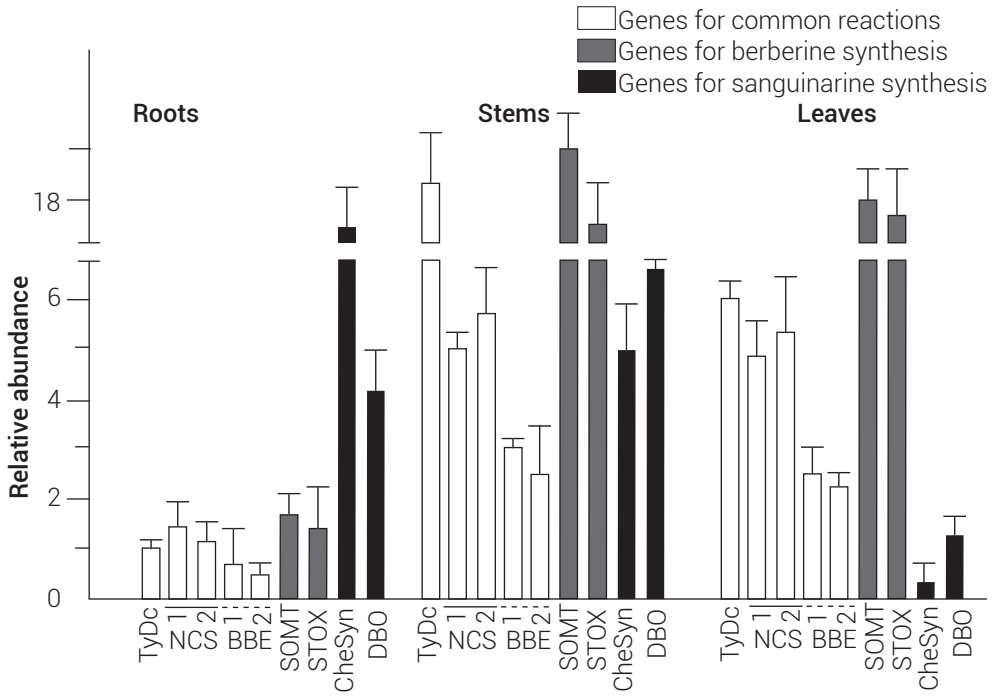


Figure 3. Distribution and relative abundance of alkaloid biosynthetic transcripts in tissues of *A. mexicana*. Average of three replications with standard error. Enzyme acronyms as in Figure 1.

DISCUSSION

In *A. mexicana*, genes coding for enzymes involved in the common reactions (TyDC, NCS and BBE) were expressed in all the analyzed tissues; however, they showed lower levels, yet higher alkaloid contents in roots than those in the aerial tissues (Figures 2 and 3). Moreover, no preferential expression of any of the NCS or BBE isoforms was noticed in any of the tissues (Figure 3), what suggests that both isoforms might function either as redundant or additive copies, rather than being associated to a differential tissue-specific expression. No differential expression of these isoforms had previously been noticed neither in developing *A. mexicana* seedlings when exposed to diverse conditions, nor *in vitro* cultures exposed to a set of chemical elicitors (Guízar-González *et al.*, 2016; Xool-Tamayo *et al.*, 2017a, b), which supports this interpretation.

Expression of the berberine-related *SOMT* and *STOX* genes (Figure 1) was found in leaves, stems and roots (Figure 3), coinciding with the presence of the alkaloid in the same tissues (Figure 2). However, as for the previous genes, lower expression was detected in roots, what suggests that some of the berberine accumulated in roots could have been mobilized from the aerial parts (Xool-Tamayo *et al.*, 2017a). Interestingly, even when *CheSyn* transcripts accumulated at the highest levels in roots, matching with the amounts of sanguinarine, significant transcript levels were also noticed in stems (Figure 3), where no sanguinarine was detected (Figure 2); such findings point towards a possible participation of stems in sanguinarine synthesis. To confirm this, a candidate gene for DBO, the enzyme involved in the last reaction (Figure 1), was included in the analysis (Xool-Tamayo *et al.*, 2017b). The corresponding transcripts accumulated mainly in stems (Figure 3), suggesting its possible participation and pointing towards the operation of a sanguinarine transport mechanism to roots.

Taken together, these data suggest that both the aerial and underground *Argemone* tissues are involved in the synthesis of its main alkaloids, regardless the site of final accumulation. Roots, displaying the major alkaloid accumulation, presented a lower transcriptional activity compared to stems and leaves (with the only exception of *CheSyn*). Moreover, stems could be involved in sanguinarine synthesis, where it does not accumulate. Although the operation of stem-to-root transport systems could account for these results, other alternatives must be considered.

Substrate flexibility has been recently described for some enzymes involved in the late reactions of BIA synthesis; for instance, a cytochrome P450 dependent oxidase (*CYP719A13*), could display both canadine- and stylopine-synthase activities (Díaz *et al.*, 2011). The products of the catalyzed reactions, canadine and stylopine, are required for berberine and sanguinarine synthesis, respectively. Cheilanthifonine, the product of *CheSyn*, is the substrate for sylopine synthase activity (Figure 1), which

is also required for the synthesis of allocryptoine and protopine. These alkaloids have been detected in extracts of aerial parts (Table 2; Díaz *et al.*, 2011) suggesting that *CheSyn* in stems and leaves could be participating in their synthesis, rather than taking part in sanguinarine synthesis; nevertheless, it should be kept in mind that only low amounts were detected. Moreover, many FAD depending oxidases from *P. somniferum* (*PsFADOX*) accept as substrates different intermediaries involved in late reactions of benzophenanthridine synthesis (Hagel and Facchini, 2013). Possible orthologous genes have been recently reported in *A. mexicana* (Hagel *et al.*, 2015), suggesting that alternative biosynthetic pathways involving the participation of gene products with similar catalytic properties could be operating in roots and aerial parts.

Finally, the operation of posttranscriptional mechanisms could also account for the discrepancies between transcript and alkaloid accumulation. Posttranscriptional controls have been pointed out as a common trend for plant specialized metabolism; however, it mainly operates as a negative modulator, down-adjusting the effects of an active gene expression (Pichersky and Lewinsohn, 2011). Interestingly, *Argemone* roots showed high sanguinarine amounts in presence of low biosynthetic related transcripts (Figures 2 and 3).

CONCLUSIONS

Alkaloid synthesis in *A. mexicana* plants seems to be a complex process, probably involving the participation of different tissues as well as multilevel regulatory mechanisms.

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