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Transcription factor Agamous-like 12 from *Arabidopsis* promotes tissue-like organization and alkaloid biosynthesis in *Catharanthus roseus* suspension cells

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Abstract

In *Catharanthus roseus*, monomeric terpenoid indole alkaloids (TIAs) are biosynthesized in specific tissues, particularly in roots, but failed to be produced by *in vitro* undifferentiated suspension cells. In this paper, we describe the impact of the root-specific MADS-box transcription factor Agamous-like 12 (*Agl12*) from *Arabidopsis thaliana* on the differentiation of suspension cells from *C. roseus*. The expression of *Agl12* is sufficient to promote an organization of suspension cells into globular parenchyma-like aggregates but is insufficient by itself to induce complete morphological root differentiation. *Agl12* expression selectively increases the expression of genes encoding enzymes involved in the early biosynthesis steps of the terpenic precursor of alkaloids. The transgenic cell lines expressing *Agl12* produced significant amounts of ajmalicine, an antihypertensive TIA that normally accumulates in *C. roseus* roots. The present paper indicates that transcription factors involved in tissue or organ differentiation may constitute new metabolic engineering tools that could help to design *in vitro* cultured cells able to produce specific valuable secondary metabolites.

Keywords: Cell differentiation; Transcription factor; Metabolic engineering; Alkaloid; *Catharanthus roseus*

1. Introduction

Plants produce a wide range of small molecules that are classified as secondary metabolites. Most of them are involved in specific ecophysiological functions such as interactions with pollinators or defence against pathogens (Pichersky and Gershenzon, 2002). Secondary metabolites belong to different chemical families and are often produced in specific organs, specialized structures or cell types. For example, roots of higher plants have been shown to synthesize a large variety of valuable secondary metabolites including alkaloids, flavonoids, and terpenoids (Flores et al., 1999). Organ and tissue specificity of secondary metabolite biosynthesis is well exemplified by the terpenoid indole alkaloids (TIAs) in *Catharanthus roseus* (L) G. Don. All TIAs produced in plants are derived from strictosidine which is formed by the condensation of tryptamine with secologanin, the indolic and terpenoid precursors, respectively (Fig. 1). This enzymatic step is

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catalyzed by strictosidine synthase (STR). Tryptamine is derived from tryptophan by the action of tryptophan decarboxylase (TDC) and secologanin is derived from geraniol via a series of enzymatic conversions, among which the first step is carried out by geraniol 10-hydroxylase (G10H) and the last step is catalyzed by secologanin synthase (SLS). Geraniol derivates from isopentenyl pyrophosphate produced through the plastidic 2-*C*-methyl-D-eythritol 4-phosphate pathway in which 1-deoxy-D-xylulose 5-phosphate synthase (DXS) catalyses the first reaction. In *C. roseus*, the anticancer dimeric TIAs vinblastine and vincristine are produced in leaves. In these organs, *in situ* hybridization studies have shown that the genes encoding specific end-pathway enzymes are exclusively expressed in specialized leaf cell types, namely the idioblasts and the laticifers (St-Pierre et al., 1999). *Tdc*, *Str* and *Sls* genes are expressed in epidermal cells, while *Dxs* and *G10h* are specifically expressed in internal phloem parenchyma cells (St-Pierre et al., 1999; Irmler et al., 2000; Burlat et al., 2004). In contrast, monomeric TIAs such as the hypotensive ajmalicine are mainly produced in roots (Mishra and Kumar, 2000). *Str* and *Tdc* were shown to be strongly expressed in roots and weakly expressed in the aerial parts of the plant (Pasquali et al., 1992). *In situ* hybridization experiments revealed that *Str* and *Tdc* are specifically expressed in most protodermal and cortical cells around the apical meristem of root tips (St-Pierre et al., 1999). These results showed that the expression of genes encoding biosynthetic enzymes of TIAs is regulated in a tissue-specific manner.

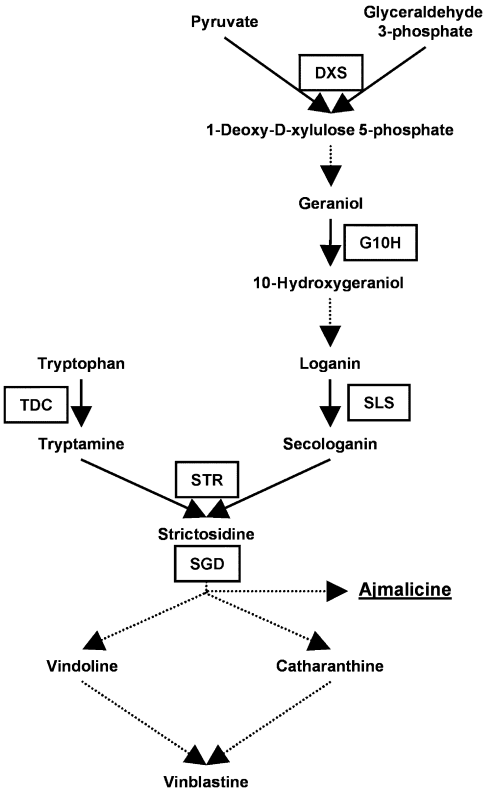

Fig. 1. Schematic representation of terpenoid indole alkaloid biosynthesis in *C. roseus*. Unbroken arrows indicate single enzymatic steps, dotted arrows indicate multiple enzymatic steps. Enzyme names are abbreviated as follows: 1-deoxy-D-xylulose 5-phosphate synthase (DXS), geraniol 10-hydroxylase (G10H), secologanin synthase (SLS), strictosidine β-D-glucosidase (SGD), strictosidine synthase (STR), tryptophan decarboxylase (TDC).

Cell suspension cultures of *C. roseus* fail to produce TIAs in growth medium. However, production can be induced by different stress signals such as auxin depletion or jasmonate addition (Gantet et al., 1998). In cell suspensions, *Str* and *Tdc* gene expression is coordinately and transiently down-regulated by auxin and up-regulated by jasmonate (Pasquali et al., 1992; Menke et al., 1999a). The jasmonate-responsive expression of these two genes and of some other genes encoding enzymes of the TIA biosynthesis pathway is mediated by AP2/ERF-domain transcription factors called ORCAs (Menke et al., 1999b; van der Fits and Memelink, 2000, 2001). These data indicate that the transient induction of TIA biosynthesis by jasmonate in suspension cells is at least partially regulated at the transcriptional level by specific transcription factors. These transcription factors are potentially useful molecular tools for metabolic engineering. They allow to coordinately induce the expression of genes encoding enzymes of specific metabolic routes leading to the production of the chosen metabolites (Gantet and Memelink, 2002).

The purpose of this work was to study whether biosynthesis of monomeric TIAs could be induced when undifferentiated suspension cells were oriented toward a root-cell or -tissue differentiation status. In an attempt to induce a differentiation favourable to biosynthesis of monomeric TIAs in undifferentiated suspension cells, we have expressed the *Arabidopsis thaliana* Agamous like 12 (*Agl12*) gene. The *Agl12* gene encodes a transcription factor displaying a MADS-box DNA binding motif (Rounsley et al., 1995). In plants, several members of the MADS-box transcription factor family are involved in flower initiation or organogenesis and in fruit development (Theissen et al., 2000). Other MADS-box transcription factors are supposed to be involved in the control of vegetative development (Alvarez-Buylla et al., 2000). The *Agl12* gene is specifically expressed in roots (Rounsley et al., 1995; Burgeff et al., 2002), with some indication of a function in root organogenesis (Burgeff et al., 2002). *Agl12* involvement in root development was confirmed in transgenic walnut somatic embryos expressing this transcription factor (Montiel and Breton, unpublished data).

Transgenic *C. roseus* cell lines expressing *Agl12* were generated by biolistic transformation. The effects of AGL12 were studied on the expression of several genes encoding enzymes of the biosynthesis pathway of TIAs and on the ability of the cell lines to produce TIAs in growth medium. The morphological organization of *C. roseus* cells expressing *Agl12* was investigated by histological microscopic observations.

2. Material and methods

2.1. Plant and cell culture conditions

Cell suspension experiments were performed with the *Catharanthus roseus* (L) G. Don C20D cell suspension. This cell line is 2,4-D-dependent and cytokinin-independent. The cells were grown in 50 ml of liquid B5 Gamborg medium, containing 58 mM sucrose and 4.5 μM 2,4-D (Gamborg et al., 1968). Cultures were started by adding 5 ml of a 7-day-old stationary phase culture to 45 ml of fresh medium and were grown in the dark at 24 °C on a rotary shaker at 100 rpm. Plant organs were harvested from 3-month-old *C. roseus* plants (variety Pacifica pink, Ducrettet Nursery, Thonon, France) grown in a greenhouse and root samples were harvested from 2-week-old hairy root culture line J1 kindly supplied by Dr. V.M. Loyola-Vargas (Ciau-Uitz et al., 1994).

2.2. cDNAs used for cell-transformation and radio-labeled molecular probes

The *Agl12* cDNA sequence (GenBank accession number U20193; Rounsley et al., 1995) was provided by Dr M. Yanofsky from University of California. The *Agl12* cDNA was excised from PCR 2.1 vector (Invitrogen) by *Eco* RI digestion and inserted into pLBR19 under the control of the double-enhanced 35S Cauliflower Mosaic Virus promoter (p35Sx2 CaMV) (Kay et al., 1987; Guerineau et al., 1992). The pGL2 plasmid carrying the *Hph* hygromycin resistance gene placed under the control of the 35S cauliflower mosaic virus promoter was obtained from Dr R. Bilang from Harvard University (Bilang et al., 1991). *Str* (GenBank accession number X61932; Pasquali et al., 1992), *Tdc* (GenBank accession number M25151), *G10h* (GenBank accession number AJ251269; Collu et al., 2001), *Sgd* (GenBank accession number AF112888; Geerlings et al., 2000) and *Rps9* (GenBank accession number AJ749993; Menke et al., 1999a) cDNAs were provided by Dr. J. Memelink from Leiden University. *Dxs* cDNA (GenBank accession number AJ011840; Chahed et al., 2000) was provided by Dr. M. Clastre from Tours University and the *Sls* cDNA (GenBank accession number L10081; Irmler et al., 2000) was supplied by Dr. J. Schroder from Freiburg University.

2.3. Biolistic transformation procedure and selection of transgenic cells

The biolistic transformation procedure was adapted from van der Fits and Memelink (1997) and Hilliou et al. (1999). Four milliliters of C20D suspension cells (4-day-old) cultured in liquid growth medium were harvested by filtration on a 50-mm diameter circular piece of Whatman 1 MM paper and transferred to solid culture medium (8 g l^{-1} agar, Sigma A-7002). Cells were grown in the dark for 24 h at 24 °C and then transformed by particle bombardment using a PDS-1000 Bio-Rad system with 1800 psi rupture disks (Bio-Rad) under reduced pressure of 30 mmHg. Ten micrograms of plasmid DNA were coated on tungsten M-25 particles (Bio-Rad) in 25 μl of 2.5 M $CaCl_2$ and 10 μl of 0.1 M spermidine. Particles were homogenized for 2 min by ultrasonication and sedimented by gravity for 15 min. Fifteen microliters of supernatant were removed and after a short sonication step, 4 μl of the remaining particle mixture were deposed on a macrocarrier disk (Bio-Rad). During bombardments, cells were placed at about 6 cm from the stopping screen. Cells were co-transformed with the pLBR19 plasmid carrying the *Agl12* cDNA under the control of a double CaMV 35S promoter sequence and the pGL2 plasmid carrying the *Hph* hygromycin resistance gene in a quantitative ratio of 4:1, respectively. Control cells were transformed with an empty expression vector and pGL2. Twenty-four hours after transformation, cells were transferred to solid culture medium containing 50 mg l^{-1} of hygromycin and placed in the dark at 24 °C. After 3 weeks, hygromycin-resistant calli were transferred individually onto solid selective culture medium in 55 mm diameter Petri dishes. Each independent transgenic cell line was grown for three additional weeks and then transferred to an initial volume of 10 ml of selective liquid culture medium on a rotary shaker at 100 rpm. The initial volume was gradually increased to 25 and 50 ml after 2 and 8 days, respectively. Hygromycin-resistant suspension cells were then maintained as described for the C20D line and analysed by Southern blot and northern blot.

2.4. Histological analysis

Five-day-old cells grown in liquid culture medium were harvested on a 30 μm nylon cloth under partial vacuum and were washed with cold water. Cell samples were fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde (v/v/v)), embedded in Paraplast and sectioned according to St-Pierre et al. (1999). Sections of 10 μm were cut with a microtome and spread on silane-coated slides. Paraplast was removed with xylene and sections were rehydrated in an ethanol series up to distilled water. Sections were stained during 15 min in 1% aqueous *O*-Safranin solution and 2 min in 1% (w/v) aqueous Astra blue solution, both followed by thorough rinsing in water. The Safranin–Astra blue technique allows the staining of polysaccharides in blue only in the absence of lignin, and lignin in pink-red both in the presence and absence of polysaccharides (Srebotnik and Messner, 1994). The samples were then dried, mounted in immersion oil under a cover slip and imaged with an epifluorescence microscope operating in bright field mode (Olympus BX51) equipped with a digital camera (Olympus DP50) and the corresponding software (Olympus analySIS).

2.5. Southern blot analysis

Genomic DNA was extracted from 25 mg of freeze-dried cells following the protocol of Weising et al. (1995). For

Southern blot analysis, 5 μg of DNA were digested with 20 units of *Sal*I or *Bam*HI (Roche Molecular Biochemicals, Meylan, France) at 37 °C during 12 h, electrophoresed in 1% (w/v) agarose gel, and transferred onto a Hybond N+ membrane (Amersham Biosciences) in alkaline conditions (0.4 N NaOH). Membranes were hybridized overnight at 65 °C in 0.3 M sodium phosphate buffer pH 8, 7% (w/v) SDS and 1 mM EDTA to [^{32}P]dATP labeled probes (2.5×10^6 cpm ml^{-1}, StripEZ^{TM} DNA labeling kit, Ambion, Austin, TX, USA). After hybridization, the membranes were rinsed, washed twice in 0.2 × SSC, 2% (w/v) SDS at 65 °C and exposed at –80 °C to X-OMAT films (Eastman Kodak Company, USA). The two enzymes used to digest the genomic DNA do not cut the AGL12 sequence but both cut the pLBR19 plasmid in only one site near the *Agl12* cloning site. In practice, each hybridizing band corresponds to an independent insertion in the genome with a cut in the inserted plasmid and a cut in the genomic DNA.

2.6. Northern-blot analysis

Five-day-old cells grown in liquid culture medium or plant organs were harvested and immediately frozen in liquid nitrogen. Total RNA was isolated from 0.5 g (FW) with the 'Plant RNeasy Mini Extraction Kit' according to the manufacturer's instructions (Qiagen). Northern blots (20 μg total RNA per lane) were hybridized with random-primed radiolabeled specific cDNAs as probes, according to Gantet et al. (1993). The *Agl12* probe was hybridized on total RNA in low stringency conditions using the hybridization solution containing 35% (v/v) formamide.

2.7. Ajmalicine determination

Ten-day-old cells grown in liquid medium or plant organs were harvested and washed with cold water. They were then deep-frozen and freeze-dried. Aliquots of 60 mg (DW) of cells or plant organs were used for ajmalicine quantification as described by Mérillon et al. (1986). The results reported were derived from three independent measurements.

3. Results and discussion

3.1. C. roseus AGL12 homologue and genes encoding biosynthetic enzymes of TIAs are expressed preferentially in roots and ajmalicine accumulates in roots

To validate our approach of using the *A. thaliana Agl12* gene, we first determined whether *C. roseus* plants were expressing a similar gene in a root-specific manner, and if so, whether its organ-specific expression pattern would match those of the genes encoding biosynthetic enzymes of TIAs and the distribution of the root-abundant alkaloid ajmalicine. A low stringency northern blot hybridization using the *A. thaliana Agl12* cDNA as a probe showed that a gene with homology to *Agl12* was strongly expressed in *C. roseus* roots, weakly expressed in leaves and undetectable in flowers and fruits (Fig. 2A). The genes encoding enzymes involved in different steps of biosynthesis of monomeric TIAs (*Str*, *Sgd*) and their terpenoid (*Dxs*, *G10 h*, *Sls*) or indolic (*Tdc*) precursors (Fig. 1) were mainly expressed in roots, to a lesser extent in leaves, and at lower level in other organs (Fig. 2A). Furthermore, ajmalicine predominantly accumulates in roots and was detected only in low amounts in leaves, flowers and fruits (Fig. 2B).

These data show that genes encoding biosynthetic enzymes of TIAs are mainly expressed in *C. roseus* roots and that this root-specific expression correlates with ajmalicine biosynthesis and with the expression domain of the *C. roseus Agl12* homologous gene.

3.2. Generation and selection of transgenic C. roseus cell suspensions expressing Agl12

The C20D cell suspension was initiated 30 years ago from leaves (Vincent Pétiard, Centre de Recherche Nestlé-Tours, France, pers. comm.), and is unable to produce alkaloids when grown in a maintenance culture medium

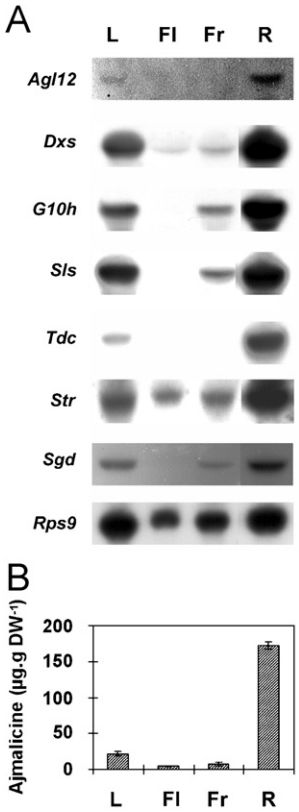

Fig. 2. Northern blot analysis of the expression of *C. roseus Agl12* and genes encoding TIA biosynthetic enzymes (A), and ajmalicine accumulation (B) in *C. roseus* leaves (L), flowers (Fl), fruits (Fr) and roots (R). Membranes were hybridized with cDNA probes indicated on the left. The *Rps9* mRNA encodes the ribosomal *C. roseus* protein S9 and was used as a loading control.

(Gantet et al., 1998). Neither differentiation of organs nor regeneration of plants from C20D line has been reported. In order to induce a differentiation compatible with biosynthesis of TIAs, the C20D cell line was co-transformed by particle bombardment with an expression cassette containing the *Agl12* cDNA driven by the strong constitutive CaMV 35S doubled promoter sequence and a plasmid carrying the hygromycin resistance gene under the control of the CaMV 35S promoter. Cell lines selected on hygromycin-containing medium were screened by Southern blot analysis for the integration of the *Agl12* expression cassette in their genomic DNA. Fig. 3 shows that the *Agl12* cDNA sequence did not cross-hybridize under the stringency conditions used with genomic DNA from untransformed C20D cells. In contrast, the four hygromycin-resistant cell lines A1–4 showed positive hybridization signals with the *Agl12* cDNA. The hybridization patterns obtained with both enzymes indicated that several copies of the *Agl12* expression cassette were integrated and that the sites of integration in genomic DNA were different for these four *Agl12* transgenic cell lines, suggesting that they resulted from independent transformation events. In addition, two hygromycin-resistant cell lines C1 and C2 transformed with the empty transformation vector were selected. These two cell lines were added to the untransformed C20D line as negative controls for further experiments. The selected lines were then cultured in liquid hygromycin-selection medium and the expression of the *Agl12* transgene was determined by northern blot analysis (Fig. 5A). Positive signals were observed at the expected size of 0.9 kb for the transgenic A lines, whereas RNAs extracted from the C20D and the C cell lines did not give hybridization signals even when low stringency conditions were used (data not shown).

3.3. Impact of Agl12 on cell suspension morphology

In the C20D and C cultures, the cells were organized in small elongated aggregates of 10–20 cells (Fig. 4). As shown for the A4 line, in A cultures, cells were organized in globular bodies that reached up to 8 mm in diameter, which in cross-section appeared to present a structure reminiscent of parenchyma-like tissue (Fig. 4). These data show that AGL12 by itself is unable to induce a complete root differentiation from *C. roseus* suspension cells. Plant organogenesis is often controlled by a set of specific transcription factors acting together. This is well illustrated by floral organ differentiation which is controlled by hetero-tetramer complexes constituted by up to four different MADS-box transcription factors (Jack, 2001). In *A. thaliana*, several transcription factors, organized in a regulatory network, have been characterized to control different aspects of the root differentiation (Montiel et al., 2004). Among them, MADS-box transcription factors have been shown to be involved in the control of root branching in response to exogenous nitrate (ARN1) (Zhang and Forde, 1998; Zhang et al., 1999) or specifically expressed in roots (AGL17 and AGL21) (Rounsley et al., 1995; Burgeff et al., 2002). These transcription factors could act in synergy with AGL12 and with other root-specific transcription factors to control root differentiation (Montiel et al., 2004). Nevertheless, these data clearly show that the AGL12 transcription factor allows the previously undifferentiated suspension cultured cells to organize themselves into globular bodies of several thousands of cells displaying parenchyma-like characteristics.

3.4. Impact of AGL12 on expression of genes encoding biosynthetic enzymes of TIAs and ajmalicine content

While comparing the C20D line, the control C lines and the A lines through northern blot analysis using probes specific to several mRNA encoding biosynthetic enzymes of TIAs, it appeared that there were no significant differences in the expression level of four genes involved in the middle steps of the biosynthesis pathway of TIAs (*Sls*, slight decrease; *Tdc*, slight increase; *Str*, no impact; *Sgd*, slight decrease), whereas genes encoding enzymes of the early biosynthesis steps of terpenoid precursors of TIAs (*Dxs*, *G10h*) were expressed at significantly higher levels in A lines compared to control C lines and the C20D line (Fig. 5A). Ajmalicine was detected at low levels in C lines and was below the detection limit in the C20D line. However, the A lines showed higher ajmalicine production (Fig. 5B). On a dry-weight basis, the ajmalicine level observed in the best transgenic producing cell line (A3) was higher than those detected in various organs of the plant and about twofold lower than in roots (Figs. 2B and 5B).

These data indicate that the AGL12 transcription factor strongly affects the transcriptional regulation of *Dxs* and *G10h* which were reported to be specifically co-expressed in the internal parenchyma cells of the leaves (Burlat et al., 2004). It is tempting to speculate that this regulation could be linked to the parenchyma-like differentiation occurring in A suspension cells expressing AGL12. This could be achieved through a direct interaction between AGL12 and MADS-box binding sequences possibly present in the promoter of these genes, or an indirect action via the activation of other transcription factors which, in turn, could activate the expression of *Dxs* and *G10h*. To test the first hypothesis, *Dxs* and *G10h* promoter sequences have to be isolated and analysed for the presence of functional MADS-box interacting sequences (Jack, 2001).

The strong induction of *Dxs* and *G10h* gene expression correlated with the production of ajmalicine by *Agl12* expressing cell lines confirms that the enzymatic steps upstream of SLS in loganin biosynthesis are limiting for biosynthesis of TIAs by undifferentiated suspension cells. This is in accordance with the fact that ajmalicine production can be observed when secologanin or loganic acid are added to the growth medium of the C20D cells (Mérillon et al., 1986; Arvy et al., 1994). Our data also show that the production of ajmalicine observed in

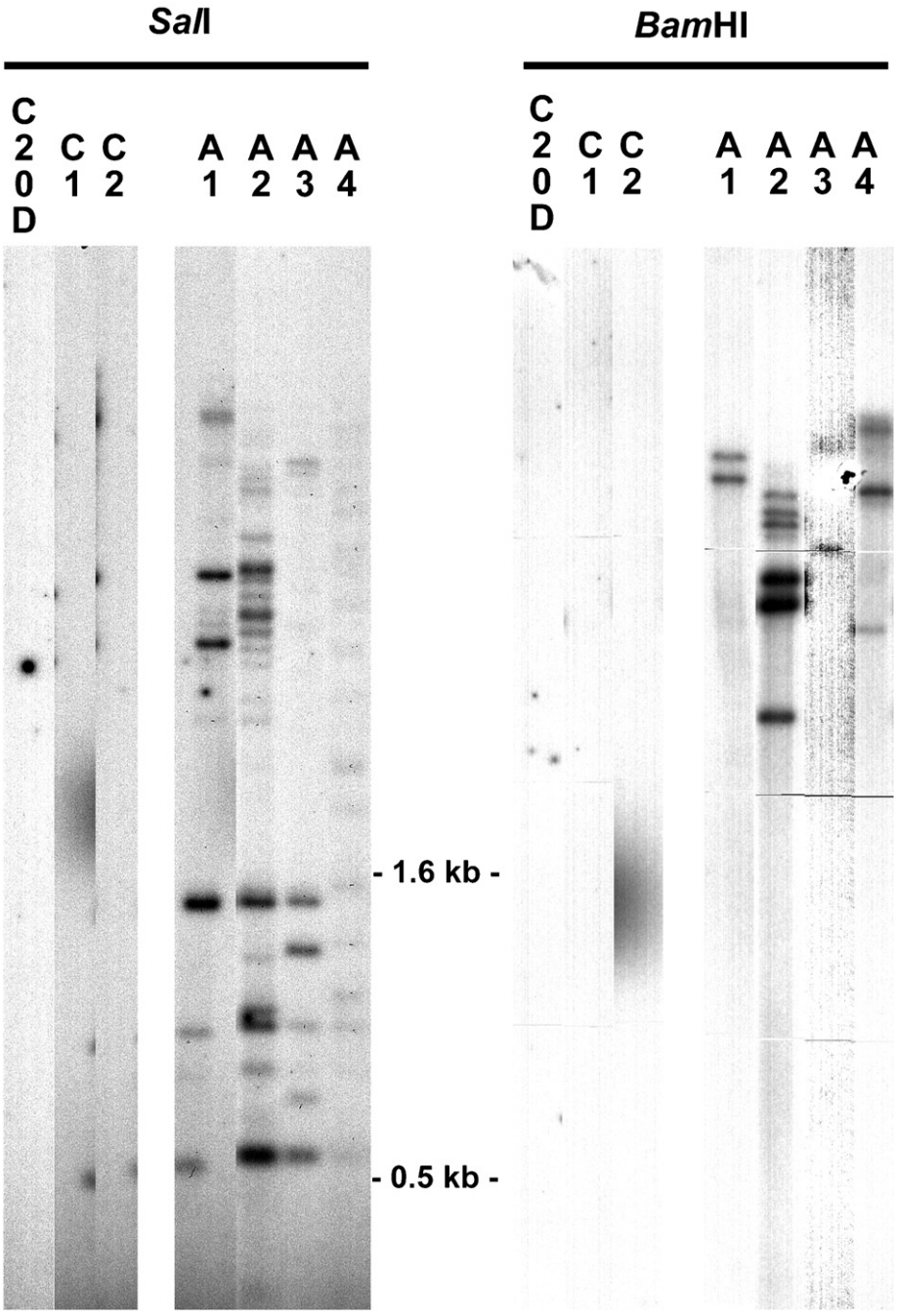

Fig. 3. Southern blot analysis of wild-type and transgenic C20D cell lines. Each lane contains 5 μg of DNA digested with *Sal*I and *Bam*HI. The blots were probed with *Agl12* cDNA. C20D: untransformed C20D strain; C1 and C2: two independent hygromycin-resistant lines transformed with the empty expression vector; A1–A4: four independent hygromycin-resistant lines transformed with an expression vector carrying the *Agl12* cDNA.

different *Agl12* expressing lines was not strictly correlated with the steady-state mRNA levels of *Dxs* and *G10h*. This suggests that other steps of the biosynthesis pathway of TIAs might be limiting. Regulation of limiting enzymatic steps could involve mechanisms taking place at the gene transcription or post-transcriptional levels including enzyme stabilization as reported for TDC (Pasquali et al., 1992; Alvarez Fernandez and De Luca, 1994; St-Pierre et al., 1999).

Low ajmalicine contents were observed in control C cell lines transformed with the empty expression vector. This could result from the multiple insertions of constitutive promoters in the cell genome associated with the cell cloning process during selection of transgenic hygromycin-resistant cell lines. Nevertheless, these levels were much lower than those observed in *Agl12* expressing cell lines, confirming the positive impact of this regulatory gene on ajmalicine production.

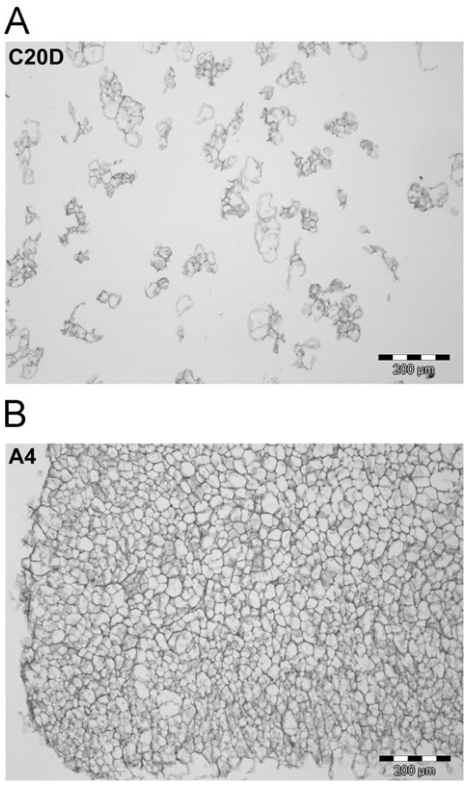

Fig. 4. Light microscopic observation of Safranin–Astra blue stained Paraplast-embedded sections of the C20D line and the *Agl12* expressing line A4 harvested on day 5 of culture.

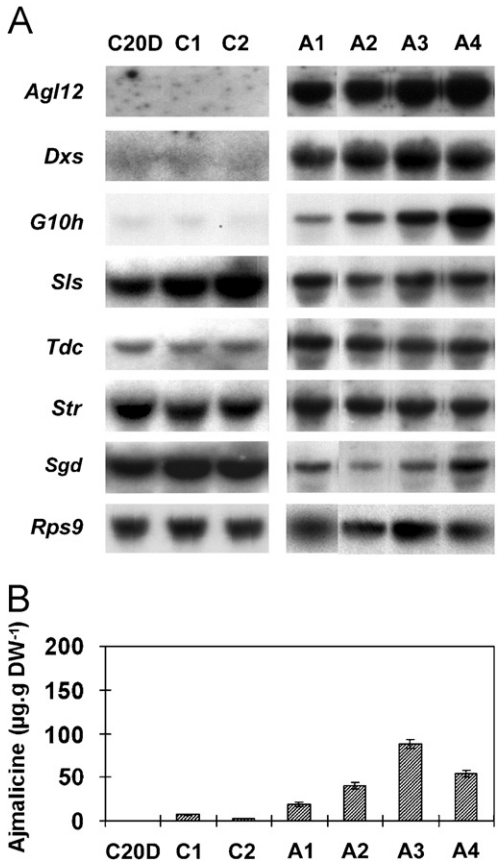

Fig. 5. Northern blot analysis of the expression of *A. thaliana Agl12* and genes encoding TIA biosynthetic enzymes (A) and ajmalicine accumulation (B) in the C20D line and in transgenic *C. roseus* cell lines. Total RNA was extracted from 5-day-old cell suspensions of the untransformed C20D line, of the C1 and C2 control transgenic lines and of the A1 to A4 transgenic lines expressing the *Agl12* cDNA. Membranes were hybridized with cDNA probes indicated on the left. The *Rps9* mRNA encodes the ribosomal *C. roseus* protein S9 and was used as a loading control. For ajmalicine determination, cells were harvested on day 10 of culture.

The present data showed that AGL12 is able to induce the production of ajmalicine in parenchyma-like organized suspension cells. This result is interesting with regard to the data previously obtained by the overexpression of ORCA3 transcription factor in *C. roseus* suspension cells (van der Fits and Memelink, 2000). ORCA3 was able to coordinately regulate the expression of a set of genes encoding biosynthetic enzymes of TIAs such as *Dxs*, *Str* and *Tdc* but failed to regulate the expression of *G10h*, resulting in a lack of secologanin and TIA production in transformed suspension cells.

4. Conclusion

These data show that *Agl12* expression is able to promote the formation of globular bodies with a parenchyma-like organization of *C. roseus* C20D suspension cells and to induce ajmalicine biosynthesis. AGL12 mainly acts on the expression level of *Dxs* and *G10h*, two genes encoding enzymes involved in the biosynthesis of secologanin which is known to be the limiting precursor of biosynthesis of TIAs in undifferentiated C20D suspension cells. Because secondary metabolite production is often associated with specialized cells, tissues or organ types, a better knowledge of transcription factors involved in the differentiation of these specialized structures may help in the future to develop new tools to engineer cell-, tissue- or organ-specific metabolism in cell suspensions.

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