Research Paper

Immunolocalization of Non-Symbiotic Hemoglobins During Somatic Embryogenesis in Chicory

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ABSTRACT

Hemoglobins are ancient O₂-binding proteins, ubiquitously found in eukaryotes. They have been categorized as symbiotic, nonsymbiotic and truncated hemoglobins. We have investigated the cellular localization of nonsymbiotic hemoglobin proteins during somatic embryogenesis in *Cichorium* hybrid leaves (*Cichorium intybus* L. var. *sativum* x *C. endivia* var. *latifolia*) using immunolocalization technique. These proteins were detected during the two steps of culture: induction and expression. In leaves, hemoglobins colocalised with plastids, which were dispersed in the parietal cytoplasm as well as in the two guard cells of a stomata, but not in epidermis cells. Upon induction of embryogenesis, in the dark, this pattern disappeared. During the induction phase, where competent cells reinitiate the cell cycle and prepare for mitosis, hemoglobins appeared initially near chloroplasts, and then in the vicinity of vascular vessels especially in the phloem and in cells surrounding the xylem vessels. When leaf fragments were transferred to another medium for the expression phase, hemoglobins were observed in the majority of the leaf blade cells and in small young embryos but not in the older ones. Hemoglobins were also detected in other leaves cells or tissues all along the process. The role of these nonsymbiotic hemoglobins during somatic embryogenesis is discussed.

INTRODUCTION

Hemoglobins (Hbs) are ubiquitous proteins present in many organisms belonging to various kingdoms including plants and animals.^{1–4} Plant Hbs were first described in the root nodules of legumes almost 60 years ago.⁵ In the 80's, Hbs from nonleguminous dicotyledons were discovered,^{6–8} and are now believed to be present in all monocotyledons and dicotyledons.^{2,8} These proteins are known as nonsymbiotic Hbs (nsHbs) because they are unrelated to the symbiotic Hbs of legumes and are not implicated in nitrogen fixation.

Physiological roles for the nsHbs are not known with certainty. Appleby et al.⁹ were the first to suggest that nsHbs could sense oxygen levels. Taylor et al.¹⁰ demonstrated that Hb cDNA was expressed in seed and root tissues of cereals under hypoxic conditions. Duff et al.¹¹ demonstrated that Hb was present during germination of barley after imbibition. Alternatively, Andersson et al.¹² suggested that Hbs might function as oxygen carriers in metabolically active tissues such as stems of soybean. Moreover, Duff et al.¹¹ supported the concept that a change in energy demand or ATP requirement is primarily responsible for Hb induction, as was previously proposed by Nie and Hill¹³ and Sowa et al.¹⁴ Such a hypothesis also finds support in the work of Lira-Ruan et al.¹⁵ However, no clear data are yet available to unambiguously assign any of these roles to nsHbs.

Plant nsHbs generally exhibit high oxygen affinities,^{2,16–18} and some authors suggest that nsHbs may be an adaptation of plants to low oxygen environments^{11,15} and may serve as oxygen sensors.^{9,19} More recently, Dordas et al.²⁰ proposed that under hypoxia the induced hemoglobin could act as a dioxygenase to control the NO level produced during this stress. Others suggested a number of other activities including reduction of metal ions²¹ and terminal oxidase activity.^{22,23}

Nonsymbiotic Hbs are cytoplasmic proteins in plants²⁴ localised in the root, aleurone and seed coat of cereals.^{10,11,25,26} Transgenic studies showed the *Parasponia* Hb promoter to be activate in the root meristem and vascular bundles in tobacco,¹⁹ as well as in vascular bundles, stems and seedling cotyledons of soybean.¹² Hunt et al.²⁷ also showed that the expression patterns in tissues of the *Arabidopsis thaliana* hemoglobin genes GLB1 (class 1) and GLB2 (class 2) are different. In fact the GLB1 gene is expressed only in germinating seedlings, whereas GLB2 gene is expressed in roots, leaves and inflorescences.

Somatic embryogenesis (SE) is assumed to be an alternative model system to study dedifferentiation and redifferentiation cellular events which occurred during SE induction stresses. Chicory offers an original model as, contrary to the carrot one, the SE is direct with no callus phase, and somatic embryos were originated from a single cell.²⁸

Chicory SE is characterized by a two-steps in vitro culture process. The first step (induction step) covers the four-day period from the dedifferentiation of a few cells and their commitment to the reactivated cell status. A few mesophyll cells, especially those localised near vascular tissues, undergo cellular modifications, which concerns cytoplasm, nucleus and the cell wall. Cytoplasm becomes dense. Parietal lenticular nucleus changes its shape, becomes spherical and voluminous; the nucleolus is also clearly visible. The nucleus is pulled toward the centre of the cell. Perinuclear cytoplasm is abundant, and cytoplasmic strands. All these steps are related to reactivating cells. The “star-shape” feature where the nucleus is in the centre of the fully reactivated cell (RC) concluded this first step. In our system, most of the RC will give rise to somatic embryos. The second step of the culture (expression step) is characterized by the RC mitosis triggering. No other mitosis occurs in mesophyll-induced tissues. The mitosis produces a cluster of embryonary cells confined into the initial reactivated cell volume and surrounded by a thick cell wall. Such a pathway of division is designated as a segmentation pattern. Except the histological studies, another approach to investigate such SE precocious events (dedifferentiation to first divisions) was to perform differential screening of a cDNA library corresponding to genes expressed during the early stages. Hendriks et al.²⁹ showed that the *Cichorium* nonsymbiotic hemoglobin gene CHI3172 was specifically expressed under somatic embryogenesis conditions, and its expression was not related to the stress caused by the in vitro culture. Recently another cDNA, CHI3160, was also identified from the same library (Scheer, personal communication).

Here, we report using anti-nHb antibodies, the immunolocalization of the nHb proteins during the two phases (induction and expression) of somatic embryogenesis in chicory leaf tissue. The time-course of their presence during the induction phase and during the expression phase is discussed in the context of their putative functions.

MATERIAL AND METHODS

Plant material. We used a hybrid of chicory called ‘474’ (*Cichorium intybus* L. var. *sativum* x *C. endivia* L. var. *latifolia*), which is an embryogenic responsive genotype (ER). Another genotype, designated as ‘Flash’ (*C. intybus* L.) is used and considered as a non-embryogenic responsive genotype (NER). Leaves from six weeks-old plantlets were used for experiments and cultured as described by Bellettre et al.³⁰ except that the induction phase was four days followed by three days of expression phase. Samples were taken during the induction phase (D₀ to D₄) and at the end of the expression phase (D₄₊₃). Fixation and embedding in resin were done immediately after collecting samples.

Production and purification of a recombinant Hb protein. Nde I and Xho I sites were introduced by PCR at the 5' end and the 3' end of the open reading frame (ORF) of the Hb 3172 cDNA, respectively.²⁹ The Nde I-Xho I restriction fragment retaining the CHI3172 Hb ORF was ligated into plasmid pET16 (Novagen) to produce a construct for the generation of the His-tagged recombinant protein. The bacterial strain BL21 of *E. coli* DE3 pLysS (Novagen) was used as expression host. To induce expression of CHI3172 Hb, IPTG (Isopropyl-β-D-thiogalactopyranoside; 0.2 mM, final concentration) was added to the bacterial

culture when cell density reached an OD₆₀₀ of 0.6. After lysozyme treatment and sonication of the bacterial culture, the recombinant protein was purified using the His Trap™ kit (Pharmacia Biotech). A white rabbit was immunized subcutaneously with 1 mg of the protein emulsified in complete Freund's adjuvant and subsequently boosted twice with 500 µg of protein. The serum was collected two weeks after the last injection, clarified and stored at -20°C until required. The specificity of the antibody was assessed by western blot analysis.

Extraction of total leaf proteins. Leaf fragments (D₀ to D₄ and D₄₊₃) were collected and stored at -80°C. Proteins were extracted from tissues as described by Boyer et al.³¹ Briefly, leaf fragments were reduced to powder, and proteins were precipitated in the extraction solution. After centrifugation the pellet was washed and the proteins were solubilized in lysis buffer. Following centrifugation, the supernatants were stored at -70°C until used for further analysis.

Denaturing electrophoresis and immunoblotting. The proteins were analysed using SDS-PAGE as described by Helleboid et al.³² Ten µL of protein sample (16 µg) were loaded. After electrophoresis, proteins were transferred onto a nitrocellulose membrane (0.45 µm, Bio Rad) by electroblotting (100V, 1 h) in a transblot cell (Bio Rad) for the immunoblot assay as described by Helleboid et al.,³² except that the primary serum dilution was 1: 2500.

Embedding and immunolocalization. Leaf fragments were fixed and embedded essentially according to the protocol described in Kronenberger et al.³³ Fragments were fixed for 4 h/RT (room temperature) in 100 mM pH 6.9 PIPES (Piperazine- N, N'-bis [2-ethanesulfonic acid]), 3.7 % (w/v) paraformaldehyde, 10 mM EGTA, 10 mM MgCl₂ and 1 mM DTT). Samples were washed in 100 mM pH 6.9 PIPES, 0.1 M Glycine, 1 mM DTT for 3 x 30 min/4°C, then in 100 mM pH 6.9 PIPES, 1 mM DTT overnight at 4°C. Dehydration was done in a graded ethanol series. Inclusion proceeded in 0.37 mL gelatine capsules (size 2, TAAB, Aldermaster, UK) with a mix of butyl- and methyl-methacrylate resin (4 Vol: 1 Vol respectively). Benzoin Ethyl Ether 0.5 % (w/v; TAAB) was used as catalyser. Polymerisation occurred at 4°C under UV light.

Sections (3 µm) were made using a Leica Microtome, and were collected in water drops on silanized-coated slides (Superfrost; 2% in acetone (v/v), 3-aminopropyl-trimethoxysilane, Fluka). Slides were stored dust free at 4°C.

For immunolocalization, resin was removed with acetone and then tissues were rehydrated in a graded ethanol series, and finally in Phosphate Buffer Saline (PBS: 137 mM NaCl, 2.7 mM KCl, 20 mM Na₂HPO₄, 1.15 mM KH₂PO₄), 5 min each bath at RT. Non-specific sites were saturated in PBST = PBS with 2 % BSA (w/v) and 0.05 % Tween 20 (v/v). The first incubation occurred with anti-Hemoglobin antibodies (1/500) in PBST, for 90 min at 37°C. After rinses in PBS (3 x 10 min at RT), a second incubation was done with goat anti-rabbit antibodies conjugated with FITC (Fluorescein IsoThioCyanate; Jackson ImmunoResearch Labs., West Grove-PA, USA), diluted 1/100 in PBST, for 90 min at 37°C. After three additional rinses in PBS, slides were mounted in Fluoromount (EMS, Fort Washington PA, USA) and stored at 4°C. Observations were made with an Olympus BH-2 microscope under ultraviolet irradiation, at $\lambda_{exc} = 480$ nm: $\lambda_{em} = 505$ nm for FITC, $\lambda_{exc} = 365$ nm: $\lambda_{em} = 420$ nm for cellulose autofluorescence, or in bright field with Nomarski interferential contrast optics. Controls were pre-immune rabbit serum or PBS as first incubation and no labelling was observed (data not shown).

Table 1 Presence (+) or absence (Ø) of nsHbs bands on the immunoblots of total leaf proteins during the SE process in ER and NER genotypes

	ER genotype		NER Genotype	
	nsHb1 18 kDa	nsHb2 18.4 kDa	nsHb1 18 kDa	nsHb2 18.4 kDa
D ₀	Ø	Ø	Ø	Ø
Induction phase				
(D ₁ -D ₂)	Ø	+	Ø	+
(D ₃ -D ₄)	+	+	Ø	++
Expression phase				
(D ₄₊₃)	+	+	Ø	++

Blots were treated with polyclonal anti-chicory nsHbs antibodies. The band corresponding to 18 kDa is related to CHI3172-Hb1 and the band from 18.4 kDa to CHI3160-Hb2.

RESULTS

Immunoblotting. The specificity of the antiserum was determined by western blot using the bacterial whole cell lysate. As shown on the immunoblot (Fig. 1A), the antiserum recognised the recombinant Hb1 (CHI3172). A minor band appears beneath the Hb band that probably corresponds to cross-reactivity with either a smaller protein, a small portion of apoHb or non-tagged Hb.

The accumulation of nsHbs during SE process was followed by immunoblot with antiserum, which recognized both nsHbs (CHI3160-Hb2 and CHI3172-Hb1). The results were summarised in Table 1 for both genotypes tested and during the two-step SE process.

In the embryogenic responsive genotype (ER, Table 1, Fig. 1B), no nsHbs forms were detected on blots at D₀ (lane 2). The nsHb1-related band (18 kDa) was detected from D₃ (lane 4) and was still present at D₄₊₃ (lane 7). The Hb2-related band (18.4 kDa) was present during the induction phase (D₁ to D₄, lanes 3 to 6), and at the end of the expression phase (D₄₊₃, lane 7).

In the non-embryogenic responsive genotype (NER, Table 1, Fig. 1C), no nsHbs forms were detected on blots at D₀ (lane 2). While the nsHb1-related band (18 kDa) was not detected during SE process, the Hb2-related band (18.4 kDa) was observed during the induction phase (D₁ to D₄, lanes 3 to 6), and at the end of the expression phase (D₄₊₃, lane 7). The intensity of this band increased particularly at D₃, D₄ and D₄₊₃.

Non-symbiotic hemoglobins are present in chicory mesophyll. Immunolocalisation of nsHbs were performed in semi-thin sections of chicory leaf (ER genotype). Four major cellular sites were identified and were not related to any SE features. Signal was observed at the periphery of the mesophyll cells (Fig. 2A), probably in the parietal cytoplasm near the plasmalemma. In a few areas dense dots were observed that possibly represent plasmodesmata. No staining was detected in the cell wall. A signal was also detected within the cytoplasm of guard cells (Fig. 2A and B). In the cytoplasm of some cells, longitudinal structures colocalized with the chloroplasts were brightly stained (Fig. 2C).

In this ER genotype, a few mesophyll cells exhibiting a voluminous nucleus and dense cytoplasm exhibited also a diffuse signal (Fig. 2C).

Finally, a signal was observed within the vascular bundle (Fig. 2D and E) especially in small cells close to metaxylem considered as parenchyma cells (arrow head), and in small phloem cells, considered as companion cells and as phloem parenchyma.

Figure 1. Immunoblot of SDS-PAGE gel corresponding to the bacterial whole cell lysate using the antibodies rose against CHI3172. MW: prestained low-range molecular weight marker proteins: lysozyme (21.8 kDa). (A) Immunoblot of SDS-PAGE gel corresponding to the proteins from leaf fragments of the embryogenic responsive genotype (ER) and the non-embryogenic responsive genotype (NER) submitted to the embryogenesis induction: day 0, 1, 2, 3, 4 (lanes 2, 3, 4, 5, 6 for both genotypes) and submitted to the embryogenesis expression: day 7 (lane 7 for both genotypes) using the Hb serum. (B,C) Hb is localised at 18.4 kDa and 18 kDa (arrow head). MW: prestained low-range molecular weight marker proteins: lysozyme (21.8 kDa).

Non-symbiotic hemoglobins are differentially present in NER and ER genotypes during SE process. In NER genotype, a signal was detected within mesophyll cells at D₀ as small and scarce dots in parietal cytoplasm (Fig. 3A). No guard cells were stained. During the induction phase, at D₃, the signal was observed in all cell-types (Fig. 3B). At the end of the induction phase (D₄), the signal seemed more diffused. The same cell-type localisations in the vascular bundle (VB) were observed and some organelles, such as chloroplasts, exhibited staining. At the end of the expression phase (D₄₊₃) a decrease of the staining intensity was noticed, particularly in the cytoplasm (Fig. 3C). In addition, staining was only observed in the parietal cytoplasm of mesophyll cells, but not in the vascular bundle or in organelles.

In the ER genotype, a signal was noticed from D₀ to D₄₊₃, as in the NER one, but the time-course was different. At D₀, dots and small linear aggregates were detected in mesophyll and guard cells. They were more abundant than in NER leaves (Fig. 3D). During the induction phase, at D₃, the signal appeared in the cytoplasm of mesophyll cells, especially those that exhibited large amount of cytoplasm and round voluminous nucleus. Such cells were noticed close to the vascular bundles (Fig. 3E and F). In these cells, the signal is localized in parietal and peri-nuclear cytoplasm. At the end of the induction phase (D₄),

the signal was also present as linear aggregates, which appear to colocalize with chloroplasts (data not shown). The staining intensity was lower than in NER leaves. At the end of the expression phase (D_{4+3}), signal was more diffused in all cell-types, but was higher than in NER (Fig. 3G).

Non-symbiotic Hbs are notably present in embryogenic- and embryony-related structures. During the induction phase (D_0 - D_4), a few mesophyll cells undergo cellular reactivation for their reentry into the cell cycle. Most of these cells will be able to give rise to somatic embryos. During this step, reactivated cells (RC) are induced and exhibit a signal in the dense parietal cytoplasm and in the strands of cytoplasm that fragment the vacuole (Fig. 4A). At the end of the induction phase (D_{4+3}), RC exhibits a central nucleus surrounded by cytoplasmic strands and the signal decreases. It was noticed that some mesophyll cells did not exhibit any nsHbs during this step of culture, but a few cells, which did not show any characteristic cytological criterion of cellular reactivation seemed to have some Hbs in their cytoplasm and around the nucleus.

During the expression phase, reactivated cells undergo embryony development via mitosis, which lead to a segmentation pathway. The signal was differentially present during the development of the embryony structures (D_{4+3}). When the embryos are small (50–60 μm by a section plane) a signal is observed in the cytoplasm and the strands of cytoplasm, which radiate from the nucleus (Fig. 4B and C). While mitosis occurs, embryony cells shrink, and no vacuole is observed. The intensity of the staining decreases, until the embryos reach about 100 μm (by a section plane) where signal was no more observed (Fig. 4D and E).

DISCUSSION

A constitutive form of nsHbs is present in mesophyll. We have observed a constitutive form of the Hb proteins at D_0 in chicory leaves. The presence of proteins and transcripts of Hb has also been previously reported in leaves of *Myrica gale* and *Arabidopsis*.^{8,34} The nature of fluorescent signal (FITC) detected in chicory leaves differed between the non-embryogenic genotype (cv. 'Flash') and the embryogenic one (hybrid '474'). Nonsymbiotic Hb is slightly expressed (1–20 μmol) in roots, meristems or germinating seedlings of monocotyledonous¹⁰ and dicotyledonous plants.^{6,19} Considering the difference in the fluorescence intensity, we suggest that nsHbs are differentially regulated in their constitutive form between NER and the ER genotypes. In our experiments, antibodies recognized both proteins originating from *CHI3172* and *CHI3160* genes; it is not possible to link the constitutive form of Hb proteins (at D_0) with a specific gene. It is difficult with light microscopy to assess if the proteins (dot features) are in the cytoplasm, near or linked with the plasmalemma, or in/associated with plasmodesmata (for bright ones). The linear aggregates colocalised with chloroplasts. We have no idea if the proteins are linked to the plastid envelope, but it is established that O_2 produced during the photosynthesis is retained as oxyHb to constitute a base for NADH oxidation.¹⁴ These authors found this Hb to be a constitutive protein. A discrepancy is observed between immunoblots and immunolocalisation, especially at D_0 . It may be due to the detection threshold of the western blot, which is too high to detect the small amount of Hbs, observed with immunolocalisation. In this last case, the amount of proteins is concentrated but they are diluted among total proteins for western blotting.

Nonsymbiotic Hbs forms are inducible during the induction phase of SE process. We have also observed two inducible forms of nsHbs in chicory: *CHI3160* (18.4 kDa, $D_{1,2}$) and *CHI3172* (18 kDa,

Figure 2. Immunolocalisation of nonsymbiotic hemoglobins in semi-thin section of ER genotype (*Cichorium* hybrid '474') leaves induced. Guard cells (gc) and the parietal cytoplasm (par cyt) of mesophyll cell were labelled. Dense dots are also observed corresponding to plasmodesmata. VB: vascular bundle. (A) Details of guard cell, a staining was observed in perinuclear cytoplasm and at the periphery of cell but not in the epidermic cell (ep c). (B) Details of mesophyll cell. A staining was observed in cytoplasm (cyt) in chloroplast (chlp) and around a nucleus (n). (C) Vascular bundle observation. (D and E) Hb was observed in small cells close to phloem (phl) and metaxylem (mXyl, arrow head) and in mesophyll cells (mc). Bar, 10 μm .

D_3 - D_{4+3}). The intensity of fluorescence varied during the culture and depended on the type of chicory. We think that this second nsHb could be induced as a response to cellular reactivation and embryony mitosis, which consumes ATP and oxygen. It has been previously observed that Hb genes are induced during various physiological phenomena.¹¹ Disappearance of the constitutive dot/linear aggregates shape at D_{4+3} could be linked to the culture in darkness, and thus to etiolation of the leaf fragments and consequently the modification of the chloroplasts structure.

Nonsymbiotic Hbs were detected during cell reactivation for SE in chicory. They were found in the cytoplasm, especially in the vicinity of the nucleus, as well as in strands of cytoplasm, which fragment the vacuole. It was noticed that the fluorescence intensity decreased at the end of the reactivation; this suggests an up and down regulation occurring during the premitosis phase. In SE process, a dedifferentiation

Figure 3. Immunolocalisation of non-symbiotic hemoglobins in semi-thin section of NER genotype (*Cichorium* cv. 'Flash') leaves induced (A–C) and in semi-thin section of ER genotype (*Cichorium* hybrid '474') during somatic embryogenesis (D–G). At D₀, Hb was detected as small and scarce dots around parietal cytoplasm of mesophyll cells (arrow head). (A) At D₃, Hb is noticed in all cell-types, particularly in mesophyll cells near vascular bundle (VB), in epidermis (ep) and in vascular bundle. (B) At D₄₊₃, dots were only observed around parietal cytoplasm (par cyt) of mesophyll cells but not in vascular bundle and in organelles (arrow heads). (C) At D₀, staining was in guard cells of the stomata (st). Some spots were observed among the limb (arrow heads). (D) At D₃, Hb was observed in the cytoplasm of mesophyll cells, in reactivated cells (RC) near a vascular bundle (VB). (E) Nomarski contrast observation in white light. (F) After 7 days of culture (D₄₊₃), the epidermis exhibits a tightly labelling. (G) All mesophyll cells have Hb in their cytoplasm. Some reactivated cells (*) were newly induced close to the vascular bundle (VB) and exhibit Hb in their nucleus. Bar, 20 µm for (A–C); 50 µm for (D); 10 µm for (E–G).

event is necessary for the commitment into SE pathways.²⁸ During precocious phases of barley germination, hydration of proteins and synthesis of proteins and nucleotides were assumed to use ATP. In this case, Hb was induced.¹¹ Cellular reactivation in chicory should also consume ATP, as cells were metabolically active. In soybean, the expression of Hb was more intense in the root elongation zone (differentiation) than in the root meristem. Andersson et al.¹² suggested that soybean Hb was not correlated with cellular division but with the

differentiation process. On the other hand, in a synchronized alfalfa cell suspension culture, the nonsymbiotic hemoglobin gene *Mhb1* is activated during the cell cycle before mitosis.³⁵ In this case, the observed activation of the *Mhb1* gene at G₂/M transition may reflect an increased metabolic activity and oxygen consumption.

The Hb gene expression could be involved in communicating signals to tissues, which are depleted of O₂.¹¹ But we should also consider that NER genotype exhibited inducible Hb, which did not correlate with any visible cellular events. This led us to propose that a part of the inducible Hb pool could be the result of in vitro culture (low O₂ tension, high osmotic pressure). In fact, on immunoblots, chicory nsHb2 (CHI3160) was observed during the first days of culture (D₁ and D₂) and this induction may be related to the culture transfer stress. This hypothesis was reinforced by the observation that the same Hb was again detected at D₄₊₃ (data not shown) after transfer in a new medium (three days), which involves a new culture stress, and when this genotype was submitted to the culture conditions used for somatic embryogenesis induction in ER one.

Figure 4. Localization of non-symbiotic hemoglobins in semi-thin section of ER genotype (*Cichorium* hybrid '474') during induction step (D₀ to D₄) and during expression step (D₄₊₃). A slight staining can be observed in mesophyll cells (MC) which increased at the beginning of the reactivation (parietal and perinuclear cytoplasm of reactivated cell: RC) and decreased at the end of reactivation (perinuclear and strands cytoplasm) when the nucleus migrates towards the centre of the cell. (A) Observation of the staining during the somatic embryos (Emb) development. The young embryos (Emb) exhibited a staining in the parietal and perinuclear cytoplasm, (B) which decrease with the embryonic mitosis in the perinuclear cytoplasm (C). When the embryo size reached 100 µm (D), no staining was observed (E). Some reactivated cells (RC) were newly induced after transfer of samples in the new medium. After seven days of culture in obscurity, chloroplasts (chlp) showed a red fluorescence typical of chlorophyll and some spots of Hb were detected in this organelle. Bar, 10 µm.

Non-symbiotic Hbs forms progressively disappeared during the expression phase of SE process. Non-symbiotic Hbs were also detected in the precocious phases of the embryo development in chicory and seems to be related to the size of the somatic embryos. It may be that O₂ consumption was different during embryos growth. In carrot, the number of somatic embryos was not affected by O₂ concentration but development was observed until heart stage,³⁶ suggesting that O₂ uptake was differentially regulated during embryo development. Moreover, Huang et al³⁷ showed that there were O₂ transfer limits when the carrot embryo size increased. Shimazu and Kurata³⁶ suggested that there was an O₂ gradient depending on the size, geometry and embryo respiratory activity.

Putative physiological roles for nsHbs during SE process. Few reports are available for nsHb location within plant tissues, or regarding their impact on O₂ consumption in vitro culture. Hypoxia stimulates the proliferation of shoot tips in *Spirea*³⁸ and is also known to alter the growth of *Catharanthus roseus* suspension cells.³⁹ Hypoxia has also been shown to prevent precocious germination and facilitate further embryo development in wheat.⁴⁰ Such a hypothesis has also been proposed during somatic embryogenesis by Kessel and Carr⁴¹ and Carman⁴⁰ in work describing the switch of balance between morphogenesis/somatic embryogenesis in the carrot. Such a balance between organogenesis and somatic embryogenesis is also observed in the chicory interspecific hybrid '474'⁴² but seems to be linked to temperature. In 1998, we demonstrated that chicory plantlets submerged in paraffin oil for 2–4 days before being subjected to the two-step in vitro culture showed an increase in number of somatic embryos produced per mm² leaf blade (unpublished results). Moreover, the induction phase occurs in the presence of 330 mM glycerol; such an osmoticum could decrease the O₂ available in the culture medium. Carman⁴³ observed such a decrease in O₂ related to the osmotic potential during wheat germination.

The last location of Hb was the vascular bundle, especially in xylem parenchyma and phloem companion cells. This was already observed in different plants.^{8,12,19,34} This observation could be linked to the necessity of communication between cells, probably for signalling hypoxic stress. In the ER genotype, this protein plays an additional role and participates in the SE process as reactivated cells and young somatic embryos needed more O₂ for their active metabolism and ATP uptake. The perivascular area in chicory mesophyll seems to be also the location of the first wave of cell reactivation, which occurs during the four-day induction phase.^{28,30}

The localization of non-symbiotic Hbs give us details on their spatial and temporal regulation during induction of SE and precocious development of somatic embryos. Further investigations with transmission electronic microscopy could help us to elucidate ultrastructural compartmentalization of the proteins as it was already described for symbiotic leghemoglobin during the infection step of *Casuarina* root nodule by *Frankia*.⁴⁴ These ultrastructural studies will complete the recent establishment of the biochemical properties of such nsHbs⁴⁵ and the demonstration of the role of hexacoordination in the regulation of affinity constants for ligand from hexacoordinate plant hemoglobins.^{46,47}

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