Nonsymbiotic hemoglobins in rice are synthesized during germination and in differentiating cell types

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Summary. Nonsymbiotic hemoglobins (ns-Hbs) previously have been found in monocots and dicots; however, very little is known about the tissue and cell type localization as well as the physiological function(s) of these oxygen-binding proteins. We report the immunodetection and immunolocalization of ns-Hbs in rice (Oryza sativa L.) by Western blotting and in situ confocal laser scanning techniques. Ns-Hbs were detected in soluble extracts of different tissues from the developing rice seedling by immunoblotting. Levels of ns-Hbs increased in the germinating seed for the first six days following imbibition and remained relatively constant thereafter. In contrast, ns-Hb levels decreased during leaf maturation. Roots and mesocotyls contained detectable, but lower levels of ns-Hbs. Split-seed experiments revealed that ns-Hbs are synthesized de novo during seed germination and are expressed in the absence of any signal originating from the embryo. Immunolocalization of ns-Hbs by confocal microscopy indicated the presence of ns-Hbs primarily in differentiated and differentiating cell types of the developing seedling, such as the aleurone, scutellum, root cap cells, sclerenchyma, and tracheary elements. To our knowledge, this is the first report of the specific cellular localization of these proteins during seedling development.

Keywords: Nonsymbiotic hemoglobin; Plant development; Immunodetection; Confocal microscopy; Seed germination; Oryza sativa L.

Abbreviations: Hbs hemoglobins; Ns nonsymbiotic.

Introduction

Hemoglobins (Hbs) are ubiquitous proteins found in bacteria, fungi, plants, protozoa, and animals (Vinogradov et al. 1993, Bolognesi et al. 1997). Plants contain two types of Hbs: the symbiotic and nonsymbiotic (ns) Hbs (Appleby 1992, Anderson et al. 1996). Symbiotic Hbs are found only in the nitrogen-fixing, nodulated dicot species and their function is to facilitate the diffusion of oxygen to the nitrogen-fixing bacteroids in the nodule (Appleby 1984, 1992). In contrast, ns-Hbs are more widely distributed and have been characterized in both monocot and dicot species (Appleby et al. 1983, Appleby 1992, Taylor et al. 1994, Anderson et al. 1996, Arredondo-Peter et al. 1997). Specific functions have not been defined for ns-Hbs.

Expression of ns-Hbs in plants appears to be primarily in metabolically active or stressed tissues. The activity of hb promoters of Parazania andersonii and Trena tomentosa was localized to the root meristem and vascular cylinder of transgenic tobacco, using GUS as a reporter (Bogusz et al. 1990). Jacobsen-Lyon et al. (1995) reported that when the Casuarina glauca hb gene was fused to GUS and transformed into Lotus corniculatus, GUS expression was found in the meristem of the root tips, the vascular stele of roots, and the parenchyma internal to the endodermis. Using Northern blot analysis, Anderson et al. (1996) detected the highest level of Hb transcripts in stems of soybeans.

Other studies also suggest that ns-Hbs are stress-related proteins. For example, under hypoxic conditions, the ns-hb gene is expressed in roots and rosette leaves of Arabidopsis thaliana (Arabidopsis) (Trevaskis et al. 1997) and the seeds of germinating barley (Taylor et al. 1994, Hill 1998). Nie and Hill (1997) found induc-
tion of the barley hb gene under low oxygen tension and high levels of CO in aleurone tissue, as well as in
the presence 10 mM nitrate. Arredondo-Peter et al.
(1997) showed that under normal growth conditions,
hb1 and hb2 from rice are expressed in leaves but that
only hb1 is expressed in roots, suggesting differential
regulation of this small gene family during seedling
development.

In monocots, the earliest expression of ns-hb genes
is observed in germinating seeds. In barley, ns-Hb tran-
scripts and protein are present predominantly in the
aleurone cells (Taylor et al. 1994). When subjected to
abiotic stress, such as low oxygen tension and high
levels of CO, the levels of ns-Hb transcripts are
markedly enhanced in isolated barley aleurone. Using
immunoblotting methods, Duff et al. (1998) have
reported the presence of ns-Hbs in excised barley
embryos, embryo-containing and embryoleaf half
seeds, and aleurone tissue.

These collective data indicate that ns-Hbs have a
role in unstressed and stressed plant tissues, although
the biochemical function(s) of ns-Hbs is still un-
Appleby et al. (1988) suggested that ns-Hbs may not
be involved in the facilitated diffusion of oxygen but
rather may function as an oxygen sensor. In contrast,
Andersson et al. (1996) suggested that ns-Hbs might
function as oxygen carriers in metabolically active
tissues, such as stems of soybean. However, no infor-
mation on the quantity of ns-Hb protein or the affinity
of soybean ns-Hbs to gaseous ligands was reported.
Ns-Hbs are now known to possess the highest reported
oxygen affinities among plant Hbs (Arredondo-Peter
et al. 1997), which leads to intriguing questions about
their function(s) in plant tissues. The high oxygen
binding affinity of recombinant ns-Hbs (r-ns-Hbs)
from several species is brought about by a moderate
association constant coupled to an extremely low dis-
sociation constant (Duff et al. 1997, Tsvanskis et al.
The very tight binding of oxygen by r-ns-Hbs does not
readily support a role in oxygen transport for these
proteins. Instead, these proteins may function in
binding of ligands such as CO or NO and/or may inter-
act with other cellular molecules (Arredondo-Peter

In all previous work on ns-Hbs in plants, mRNA
transcripts and in some instances protein content have
been determined in tissue extracts. However, to under-
stand the function(s) of these proteins in plants, spe-
cific cell type localization is needed to define the role
of ns-Hbs.

We have studied the temporal and spatial distribu-
tion of ns-Hbs in rice tissues by immunoblotting and
confocal laser scanning microscopy. These proteins
were immunodeected in all tissue types examined
during the first two weeks following germination and
were immunolocalized in a number of cell types during
germination, with specificity to the aleurone, scutel-
lum, root cap, and in differentiating tracheary ele-
ments. We have also determined that up-regulation of
ns-Hbs during rice seed germination does not require
an embryonic signal.

Material and methods

Plant growth

Rice (Oryza sativa cv. Jackson) seeds were germinated on paper
towels in a greenhouse at 22 °C with light and dark periods of 16 h
and 8 h at 950 microwatts/m² s. Plants were watered daily with
tap water.

For half-seed experiments, dry, ungerminated rice seeds were
slid with a razor blade into embryo-containing and embryoleaf
halves, incubated separately in petri dishes on moist paper towels in
the dark, and wet daily with either distilled water or 5 μM cyclo-
ximide (Jiao et al. 1991).

Antibodies to rice r-ns-Hb1 and immunoblotting of native rice Hbs

Recombinant rice ns-Hb1 was purified according to Arredondo-
Peter et al. (1997). Polyclonal antibodies were raised against this r-
s-Hb1 in rabbits by the Antibody Core Facility of the University
of Nebraska-Lincoln. Serum titers were analyzed by Western blot-
ting techniques (Penheiter et al. 1997).

Rice seeds were germinated on filter paper soaked with distilled
water. Germinating seeds and young seedlings were harvested 2, 4,
6, 8, 10, 12, and 14 days post germination. When possible (≤4 days),
seedlings were separated into root, seed, mesocotyl, and leaf tissues
and were immediately frozen in liquid N2. Frozen plant tissue was
either processed immediately or kept at -80 °C until used. Plants
were ground in liquid N2, and resuspended in 20 mM Tris-HCl, pH
8.0, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was
squeezed through one layer of cheesecloth and centrifuged at
16,000 g for 10 min. The resulting supernatant fraction was analyzed
for protein content by the BCA protein assay (Pierce Chemical
Co.). 20 μg of total soluble protein was precipitated using 5%
trichloroacetic acid and the pellet was washed thrice with cold 100%
ethanol. Pellets were resuspended in Laemmli sample buffer and
heated at 95 °C for 3 min (Laemmli 1970) before separation on 13%
sodium dodecyl sulfate-polyacrylamide gels. The resolved polypep-
tides were electroblotted onto a nitrocellulose membrane and
blocked with Tris-buffered saline with 0.05% (v/v) Tween 20 sup-
plemented with 3% (w/v) nonfat dry milk. Western blotting was per-
formed with primary antibody raised against recombinant rice
ns-Hb1 (0.5 ng/ml) and secondary antibody of goat anti-rabbit
immunoglobulins G peroxidase (1:4 ng/ml Sigma). Blots were devel-
oped with CL-APR substrate system (Pierce Chemical Co.) and
detected by using Biomax ML imaging film (Kodak).
Localization of ns-Hbs in rice tissues

Rice tissues were collected at various developmental stages and were frozen in OCT compound (Tissue-Tek) at -20°C. Frozen sections (ca. 8 μm thick) were cut on a cryostat microtome (Leica, CM-1900) and collected on poly-tyram slides (Sigma). Slides were first treated in 100% methanol for 5 min at -20°C and washed for 2 min in PBS (phosphate-buffered saline: 137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.2, at 25°C). They were then incubated in 3% (w/v) bovine serum albumin (BSA) and PBS-0.05% (v/v) Tween 20 (PBST) for 45 min at 25°C. Primary antibody against anti-recombinant ns-Hb1 was diluted to 8 ng/ml in PBST containing 1% BSA; the negative control used was preimmune serum. Slides were incubated at room temperature for 2 h, washed twice in PBST, and then in secondary antibody consisting of Cy-2-conjugated goat anti-rabbit immunoglobulin G (Jackson Immunoresearch) (300 ng/ml in PBST containing 1% BSA), and the slides were incubated in the dark for 1 h. Samples were then washed twice in PBST and once in PBS, mounted, and examined with a confocal laser scanning microscope (Bio-Rad MRC-1024ES). Images were collected and analyzed by Bio-Rad LaserSharp (V.3.3) software.

Results

Detection of ns-Hbs in cell extracts

Immunodetection of ns-Hbs in germinating rice seedling tissue was examined with antibodies raised against rice r-nHb1 at 2, 4, 6, 8, 10, 12, and 14 days post imbibition. These antibodies reacted equally to r-nHb1 and r-nHb2 (Fig. 1A), indicating their specificity for rice ns-Hbs. The antibodies also did not cross-react to proteins other than ns-Hbs in rice extracts, indicating their utility for tissue localization experiments. To analyze the relative amounts of ns-Hbs in soluble tissue extracts from germinating rice seedlings (Fig. 1B-F) signals attributable to ns-Hbs in tissue extracts were compared to the signal observed for 50 ng of r-nHb2 which was included on each blot as a standard. In dry seeds, ns-Hbs were barely detectable (Fig. 1B). By 2 days after imbibition, ns-Hbs were detectable, and increased in abundance over the next 4 days. These data indicate that ns-Hb synthesis is apparently up-regulated during rice seed germination (Fig. 1B). Under our conditions of growth, the seminal root and the coleoptile had emerged from the seed at 4 days after imbibition. By 6 to 8 days, the primary leaf was present and the embryo contained the organs associated with a normally developing rice seedling, including the scutellum, coleoptile, coleorhiza, radicle, and vascular procambium (T. Jones and Rost 1989). Eight days after imbibition, the rice seedlings were large enough to separate into root, seed fraction (seed coat, cotyledon, and coleorhiza), mesocotyl, and leaf. As shown in Fig. 1C, ns-Hb levels in root tissue were low, and blot had to be overdeveloped to detect a signal. In the seed fraction, immunoblotting detected relatively greater amounts of ns-Hbs between 8 and 14 days as compared to the earlier stages (Fig. 1D). Ns-Hbs were also present in 8- to 14-days-old mesocotyl tissue, but at levels lower than in other tissues except leaves (Fig. 1E). The amounts of ns-Hbs in leaf extracts were apparently much lower than the 50 ng r-nHb2 standard and decreased with increasing age of the leaf tissue (Fig. 1F). Blots of the different tissue extracts were developed for varying lengths of time to obtain signals corresponding to the ns-Hbs. Thus the intensity of the signal arising from the 50 ng r-nHb2 standard included in each blot was important for determining the relative abundance of ns-Hbs in tissue extracts. Our data indicate that ns-Hbs are present in varying amounts in the different tissues during normal rice seed germination, and suggest that there may be age-dependent regulation of Hb levels in maturing tissues such as the leaf.
Immunolocalization of rice ns-Hbs by confocal microscopy

Ns-Hbs in specific rice tissues

To determine the specific cellular distribution of ns-Hbs in rice seedling tissues, immunolocalization of ns-Hbs was performed on cryofixed fresh tissue sections. However, the starchy nature of the rice seed made it difficult to obtain unfragmented sections from germinating seeds prior to 3 days post imbibition. Although we analyzed many sections obtained from varying tissues at different times during the germination process, we have selected micrographs that are representative of ns-Hb localization in the different rice tissues. Paired confocal images of specific regions of tissues were collected from adjacent sections as shown in Fig. 2. Ns-Hbs were found in specific cell types throughout the rice seedling during the first two weeks of germination. For example, a section of a 4-day-old seedling through its radicle shows ns-Hb protein is present primarily in the root cap cells (Fig. 2A–C). As seen in these images of the root cap, the ns-Hbs are apparently in the cytoplasm, as determined by the position of the nucleus, observed by an elliptical shadow within each cell. The lack of a signal peptide on rice Hb1 and Hb2 (Arredondo-Peter et al. 1997) also supports this conclusion. The aleurone and scutel-
lum, which play a key role in signaling and hydrolytic enzyme secretion during the onset of germination, showed the presence of ns-Hbs, as seen in Fig. 2D–F. The aleurone cells are characterized by their columnar appearance and the scutellum is the adjoining tissue. Distribution of ns-Hbs in this region is shown in a representative micrograph of an oblique section from a 6-day-old germinating seedling. In addition, ns-Hbs were found in a number of tissues of the leaf, including the sclerenchyma (Fig. 3G–I).

Ns-Hbs in the vasculature of rice seedlings

Immunolocalization of ns-Hbs by confocal laser scanning microscopy led to the detection of ns-Hb expression throughout the vasculature of rice seedlings (Fig. 3). Ns-Hbs were detectable in vascular tissues as early as 4 days after imbibition and were found in all developing vascular tissues examined by this technique. Specifically, ns-Hbs were localized in differentiating xylem cells, as shown in sections of the root and mesocotyl from 10-day-old seedlings (Fig. 3A–C, D–F). The ns-Hb proteins appear to be localized primarily in the xylem. Additionally, ns-Hbs were detected in the vasculature of the leaf and in particular the vasculature next to the sclerenchyma (Fig. 3G–I).

The recurrent immunolocalization of ns-Hbs in the vasculature of root, mesocotyl, and leaf tissues raises questions about the function of these proteins. The fact

Fig. 3A–I. In situ immunolocalization of ns-Hbs in vascular bundles of rice tissues. Rice tissues were treated as described in the legend to Fig. 2. A–C 10-day-old root vasculature. A Phase image of an oblique section; B positive test; C negative control. Ns-Hbs are immunolocalized to the xylem as indicated by the arrows. D–F 10-day-old mesocotyl vasculature. D Phase image; E positive test; F negative control. G–I 10-day-old leaf vasculature. G Phase image; H positive test; I negative control. Ns-Hbs are immunolocalized to the xylem as indicated and in the sclerenchyma sheath as indicated by the arrows. XY Xylem, SS sclerenchyma sheath
that ns-HBs were found specifically in the xylem indicates that these proteins may have a particular role in the metabolism accompanying the differentiation of the xylem.

Ns-HBs in differentiating tracheary elements

Differentiating xylem cells can be distinguished morphologically from nondifferentiating cells by the radial thickening of the cell walls (Fig. 4). To determine in which xylem cells the ns-Hb proteins accumulated, the immunolocalization micrographs were overlaid on the phase micrograph of the same image (Fig. 4). As seen in the representative micrographs of 12- (Fig. 4A) and 6-day-old mesocotyl (Fig. 4B), ns-HBs were predominantly localized in cells that contain radial thickening of the cell walls. Ns-HBs were also observed in parenchyma cells adjacent to the cells containing radial cell wall thickenings. We are uncertain if these parenchyma cells were in an early stage of differentiation into tracheary elements. However, proteins were not detected in fully mature xylem cells, indicating that ns-HBs are present at distinct stages of the xylem differentiation process. While the exact stage of xylo-genesis of the cells shown in Fig. 4 is unknown, the localization of ns-HBs is a first indicator that these proteins are involved in the xylo-genesis process.

Synthesis of ns-HBs does not require the embryo upon imbibition

On the basis of the finding that ns-HBs were synthesized in the aleurone cells of barley (Taylor et al., 1994; Duff et al., 1998) and rice (this work), we were interested in understanding if the embryo was required for expression of ns-HBs in these cells. Dry rice seeds were separated into embryo-containing and embryoless halves, imbibed in the presence or absence of cycloheximide, and probed for ns-Hb levels by immunoblotting. By 3 days of imbibition, both halves showed the presence of ns-Hbs, but with more protein in the embryoless half (Fig. 5, lanes 3 and 4). This indicated that the embryo was not required for ns-Hb synthesis. When the embryoless and embryo-containing halves were incubated in the presence of 5 μM cycloheximide, a eukaryotic cytoplasmic protein synthesis inhibitor, immunoblots showed less protein in both seed halves after 3 days of incubation (Fig. 5, lanes 5 and 6). However, considerably more ns-HBs were now found in the embryo-containing half as compared to the embryoless half.

Fig. 4A, B. In situ immunolocalization of ns-Hbs in differentiating xylem cells. Immunolocalization techniques were described in the legend to Fig. 2. Phase micrograph and positive micrograph were overlaid by Adobe Photoshop 5.5. A Positive and phase overlay of 12-day-old mesocotyl tissue; B Positive and phase overlay of 6-day-old mesocotyl tissue; Ns-HBs are immunolocalized in differentiating xylem cells, as distinguished by the radial cell wall thickenings (see arrows).

Fig. 5. Protein immunoblot analysis of rice seeds grown in the presence or absence of cycloheximide. Rice seeds were sliced into embryo-containing and embryoless halves and germinated in the presence or absence of 5 μM cycloheximide. 30 μg of total soluble protein was separated by 13% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analysis of ns-Hbs was as described in the legend to Fig. 1. 1 Dry, embryoless; 2 dry, embryo-containing; 3 3 days' imbibed with or without cycloheximide, embryoless; 4 3 days' imbibed without cycloheximide, embryoless; 5 3 days' imbibed with cycloheximide, embryo-containing; 6 3 days' imbibed with cycloheximide, embryo-containing. The r-Hb standard was 50 ng of rHb2.
Discussion

Using immunolocalization, ns-Hbs were identified in primarily differentiated and differentiating cell types, including the aleurone, scutellum, root cap cells, sclerenchyma, and tracheary elements. Dry, ungerminated rice seeds contained very low amounts of ns-Hbs and levels increased during germination as observed by immunochemical analyses of soluble proteins from various tissues (Fig. 1B-F). The increase in ns-Hb protein during seed germination appears to be due in part to de novo synthesis, as it is inhibited by the addition of cycloheximide upon seed imbibition (Fig. 5). However, signals originating in the embryo do not apparently control the production of ns-Hbs. These data corroborate and significantly extend earlier studies performed with barley (Taylor et al. 1994, Nie and Hill 1997, Duff et al. 1998). During seed germination and early seedling development, ns-Hb proteins were present in roots, seeds, mesocotyl, and leaves. However, there appeared to be a down-regulation of ns-Hb synthesis or up-regulation of protein turnover in maturing leaves.

Utilizing confocal laser scanning microscopy, ns-Hbs were immunolocalized in specific cell types; namely the aleurone, scutellum, sclerenchyma, root cap, and developing xylem (Fig. 2-4). Seed germination and early seedling development are processes accompanied by the programmed depletion of stored seed reserves. Typically, during germination of cereal grains such as rice, the aleurone and cells of the scutellum play a central role in mobilizing reserves (Rad and Maeda 1987). These two tissues are the principal sites for the conversion of starch into sucrose, which is then transported into the embryonic axis for subsequent metabolism (Nomura et al. 1969, Briggs 1972). With the onset of germination, new cell types are formed which take on specific roles for the growth and maintenance of the plant. These include gravi-sensing root cap cells, which differentiate from peripheral root cap cells into border cells and eventually slough off (Woo and Hawes 1997), and the cells of the vasculature and tissue such as sclerenchyma.

Analyses of different plant species transformed with a variety of ns-promoter-gus fusions have indicated that these promoters will drive gus expression in different tissues. Root-nodule-specific promoters appear to maintain this specificity in transformed plants (Andersson et al. 1997, Francke et al. 1998, Strzyzki et al. 2000). In nonnodulating transgenic plants or plants transformed with ns-hb-promoter-gus fusions, GUS activity was principally detected in the vascular bundles, roots and root cap cells (Bogusz et al. 1990, Jacobsen-Lyon et al. 1995, Francke et al. 1998, Strzyzki et al. 2000). Our observations of ns-Hb proteins in root cap cells and the vasculature are consistent with these earlier studies, and have for the first time demonstrated that native Hb proteins are indeed present in these tissues.

Localization of ns-Hbs in differentiating xylem is particularly interesting. Our studies strongly suggest that ns-Hbs are synthesized during an early stage of tracheary-element differentiation. However, ns-Hb signals were also detected in cells at a more advanced stage of xylogenesis, possibly due to sequestration of proteins within a cellular compartment (for example, cell walls) which delays their eventual degradation by proteases released during the differentiation process. Xylogenesis is a complex cytodifferentiation process in which a distinct metabolism has been documented for specific stages (Fukuda 1996, A. Jones 2000). It is unknown at what stage(s) of xylogenesis ns-hbs genes are expressed. The availability of elegant in vitro and in vivo systems to study xylogenesis (Fukuda and Komamine 1980, Fukuda 1996, Jones 2000, Roberts and McCann 2000) will be an asset to the future determination of the role of ns-Hbs during this process.

Previously, ns-Hbs have been detected in diverse tissues under stressed and unstressed conditions (Andersson et al. 1996, Arredondo-Peter et al. 1997, Trevaskis et al. 1997, Hill 1998). For example, in 6-week-old plants, the ns-hb1 and ns-hb2 genes are differentially expressed (Arredondo-Peter et al. 1997), indicating tissue-specific regulation. It is conceivable that ns-Hbs have multiple roles in the plant, which are determined by the specific gene product, the levels of functional protein, and the specific sites of protein accumulation. Seregelyes et al. (2000) have shown that cultured alfalfa cells synthesize an ns-Hb under hypoxia that is apparently nuclearly localized. This is interesting, since all plant ns-Hbs reported to date lack a nuclear-localization signal and appear to be cytoplasmic. However, ultrastructural procedures may unmask sites of protein localization not evident at lower levels of resolution.

Ns-Hbs may be involved as oxygen scavengers or in oxygen signaling (Appleby 1992, Goodman and Hargrove 2001). However, compared to symbiotic Hbs, which are found in concentrations that range from 1 to 3 mM (Appleby 1984), ns-Hbs are found in much
lower concentrations (Duff et al. 1998, Hill 1998; this work). In addition, given the in vivo localization of ns-Hbs in metabolically active tissues and the very high affinity for oxygen binding of several r-ns-Hbs, it is unlikely that they function as oxygen scavengers (Arredondo-Peter et al. 1998, Hill 1998). Both Arredondo-Peter et al. (1998) and Hill (1998) have suggested that plant ns-Hbs could act as NO scavengers, in a similar manner to those of the bacterial flavohemoglobins, which have been documented to possess nitric oxide dioxygenase properties (P. Gardner et al. 1998, A. Gardner 2000). However, ns-Hbs lack a flavin domain, which would be required for functioning as an NO dioxygenase. It has also been suggested that there is a general function for ns-Hbs as an oxygenase by oxidizing NADH in conjunction with a flavoprotein in order to maintain the energy demands of the cell (Arredondo-Peter et al. 1998, Hill 1998). Conceivably, such interactions could occur at the dimer interface of differently ligated ns-Hbs (Goodman and Hargrove 2001).

On the basis of the nature of ns-Hb expression in specific rice cell types we propose that these proteins likely play a key role in a particular metabolic function(s). We have observed ns-Hbs so far only in terminally differentiated or terminally differentiating tissues such as the aleurole, root cap cells, or differentiating xylem. All of these tissues have inherently different cell-specific metabolism but need to maintain this metabolism until no longer required by the plant (for instance, the aleurole) or have become terminally differentiated (xylem). It is possible that ns-Hbs prevent an early demise of these cells or are part of a pathway that permits the orderly progression of cell development. For instance, if they were found in all rice tissue and cell types it would be more likely that they played a general “housekeeping” role; however, the nature of our findings indicates that, indeed, there may be a specific developmental function(s) of the ns-Hbs in vivo.

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In vivo $^{19}$F NMR chemical-shift imaging of Ancistrocladus species

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Dedicated to Professor Manfred Christl on the occasion of his 60th birthday

**Summary.** $^{19}$F nuclear magnetic resonance (NMR) imaging and $^{19}$F NMR chemical-shift imaging ($^{19}$F CSI) have been used to localize fluorinated compounds administered to stems of Ancistrocladus heteromorphus and A. abbreviatus for the elucidation of biosynthetic pathways in living plants. This first application of $^{19}$F CSI on plants proved CSI to be a valuable technique for mapping fluorinated molecules in plants. Exemplarily using trifluoracetate as a model compound allowed to select appropriate feeding methods and to optimize both concentration and duration of the application to the plant. The time course of the uptake and distribution of trifluoracetate was monitored by both $^{19}$F imaging and $^{19}$F CSI. Fluorinated metabolites formed by uptake of 3-fluoro-3-deoxy-D-glucose were detected with $^{19}$F CSI.

**Keywords:** Fluorine nuclear magnetic resonance imaging; Fluorine chemical-shift magnetic resonance imaging; Ancistrocladaceae; Trifluoracetate; 3-Fluoro-3-deoxy-D-glucose; In vivo nuclear magnetic resonance.

**Abbreviations:** 3-FDG 3-fluoro-3-deoxy-D-glucose; CSI chemical-shift imaging; NMR nuclear magnetic resonance; SNR signal-to-noise ratio; TFA trifluoracetate.

**Introduction**

In vivo nuclear magnetic resonance (NMR) methods, well established in medical and pharmacological research, gain importance in investigations of plants (Chudek and Hunter 1997, Ratcliffe 1994). The non-invasive character of these techniques enables the acquisition of NMR data from living plants revealing anatomical and physiological parameters like water distribution (Connelly et al. 1987, Gidwell et al. 1999, Kuchenbrod et al. 1995, Mc Fall and Johnson 1994) or flow velocities (Rokitta et al. 1999a, b). Biochemically relevant data are obtained by monitoring the distribution of primary and secondary metabolites (Heidenreich et al. 1998; Meininger et al. 1997; Metzler et al. 1994, 1995; Wolfe et al. 2000; Ziegler et al. 1996). The overwhelming majority of published work concentrates on the ubiquitous $^1$H nucleus carrying the NMR information (Chudek and Hunter 1997, Ratcliffe 1994). In particular, metabolic studies, by contrast, are often based on nuclei like $^{13}$C, $^{19}$F, or $^{31}$P. Due to a wider range of chemical-shift differences, their NMR spectra exhibit less signal overlap than in vivo proton NMR spectra, where interpretation often fails because of overlapping signals from different protons in similar environment. Moreover, heteronuclei can be used for more specific investigations as their natural occurrence is relatively poor, allowing spectroscopical detection of administered compounds with almost no disturbing background signals. Furthermore, the NMR-active isotope $^{19}$F is present at 100% natural abundance, a very favorable situation compared to carbon, where only 1% of its naturally occurring isotopes consists of the NMR-active $^{12}$C nucleus and 99% is NMR-invisible $^{13}$C, necessitating enrichment processes or long measuring times for acquiring carbon NMR signals. Combined with the very high NMR sensitivity of 83% compared to the $^1$H nucleus, fluorine bears ideal properties for permitting the successful application of $^{19}$F NMR spectroscopy and imaging for the detection of fluorine-labelled substances (Berkowitz et al. 1990) and for the use of fluorinated compounds as NMR contrast agents (Wyrwicz et al. 1985). In spite of these apparent ben-