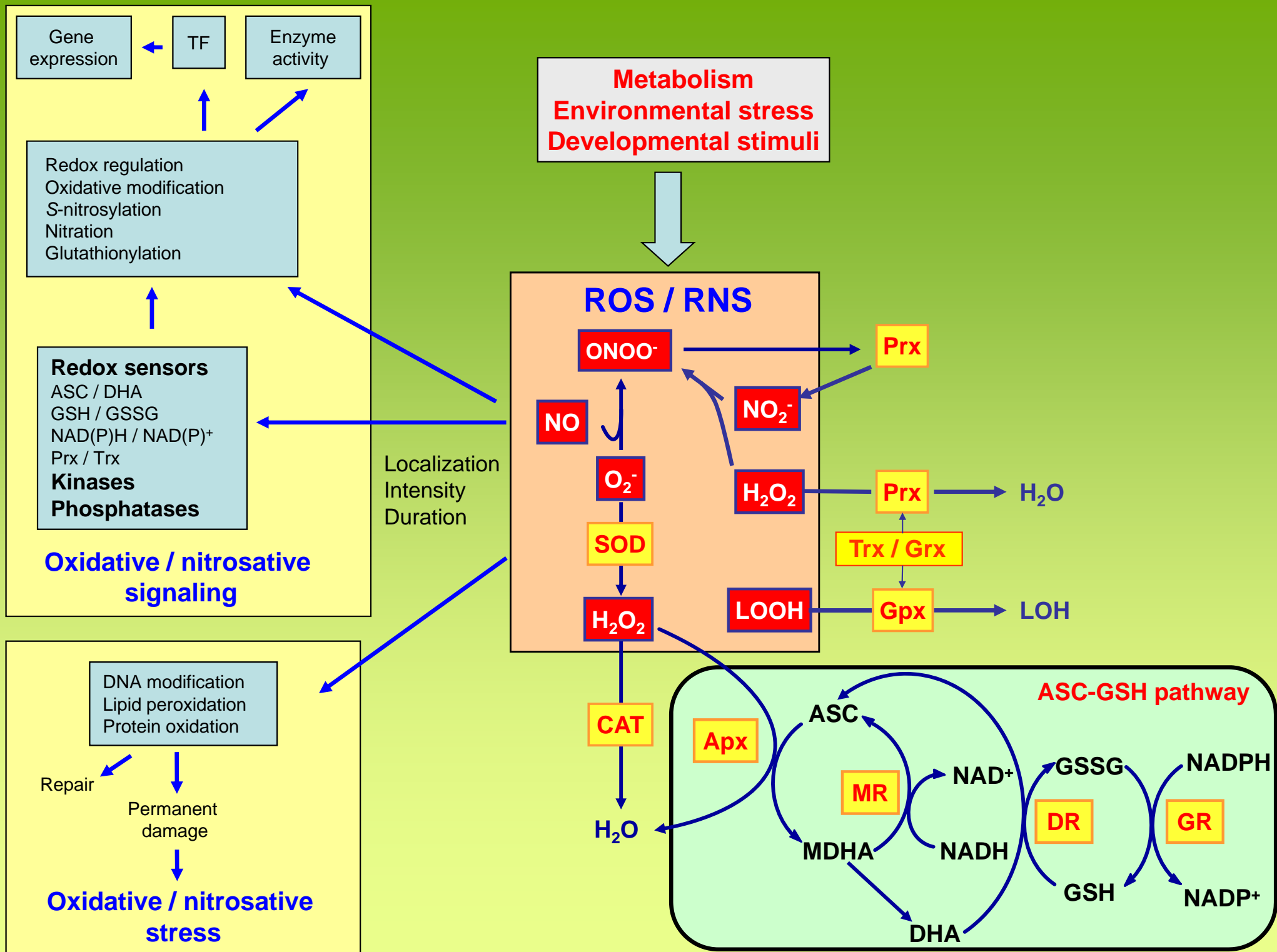




**Reactive Oxygen/Nitrogen Species and  
Antioxidants in Legume Nodules**

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Consejo Superior de Investigaciones Científicas  
Zaragoza (Spain)*



## Bacteroid

MnSOD  
CAT  
 $\gamma$ ECS  
GSHS

## Cytosol

CuZnSOD<sub>c</sub>  
APX<sub>c</sub>, MR<sub>c</sub>  
DR<sub>c</sub>, GR<sub>c</sub>  
GSHS, hGSHS  
PrxII B/C

## Peroxisome

MnSOD  
CAT  
APX<sub>px</sub>, MR<sub>px</sub>  
DR<sub>px</sub>, GR<sub>px</sub>

## Plastid

Ferritin  
APX<sub>p</sub>, MR<sub>p</sub>  
DR<sub>p</sub>, GR<sub>p</sub>  
CuZnSOD<sub>p</sub>  
FeSOD<sub>p</sub>  
 $\gamma$  ECS  
hGSHS  
Prx2CA, Prx2CB  
PrxQ, PrxII E

## Nucleus

CuZnSOD  
FeSOD  
Prx1C

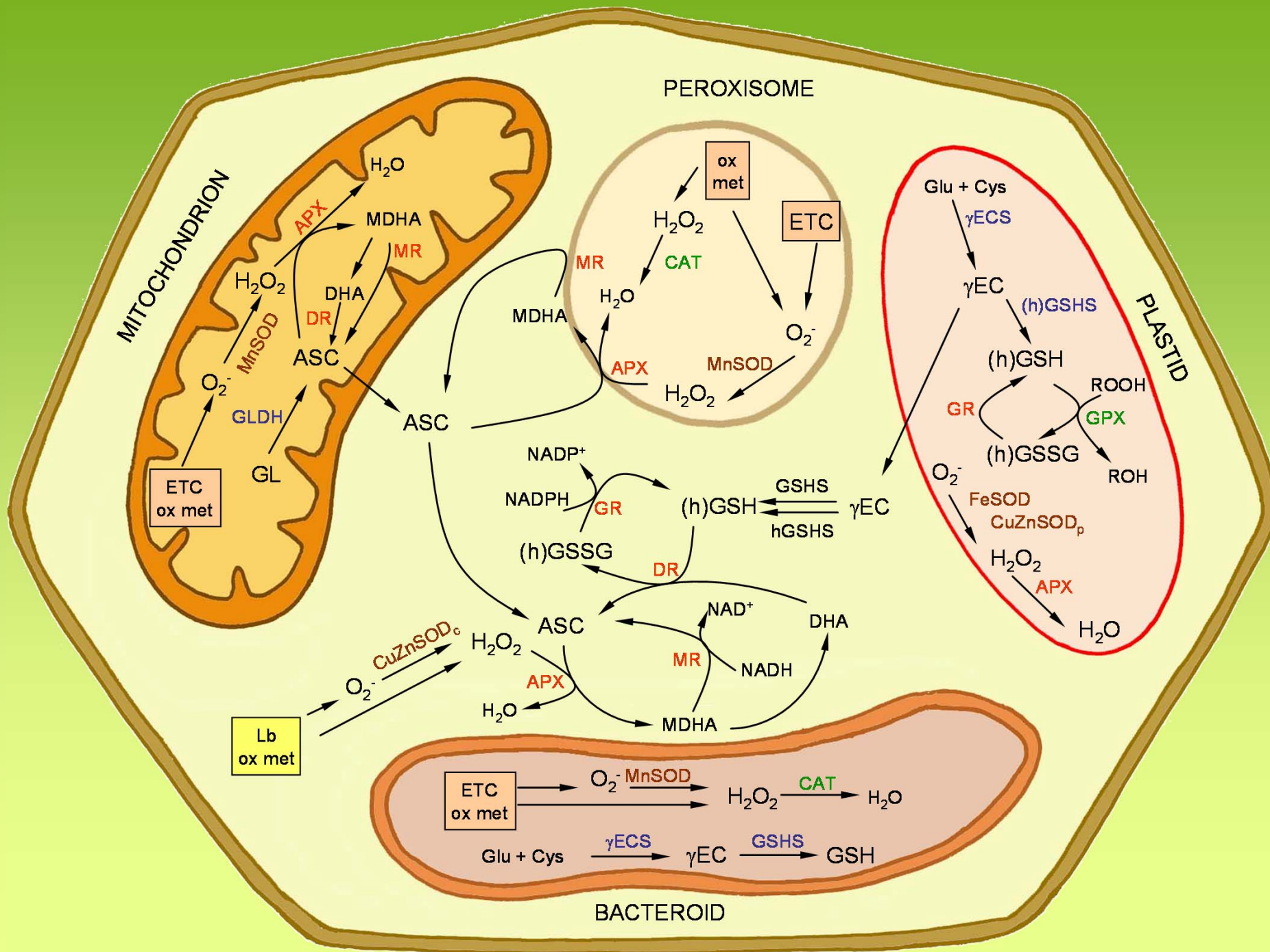
## Mitochondrion

MnSOD  
APX<sub>m</sub>, MR<sub>m</sub>  
DR<sub>m</sub>, GR<sub>m</sub>  
PrxII F

**Abbreviations:** APX, ascorbate peroxidase; CAT, catalase;  $\gamma$ ECS,  $\gamma$ -glutamylcysteine synthetase; DR, dehydroascorbate reductase; GR, glutathione reductase; (h)GSHS, (homo)glutathione synthetase; MR, monodehydroascorbate reductase; PRX, peroxiredoxin; SOD, superoxide dismutase

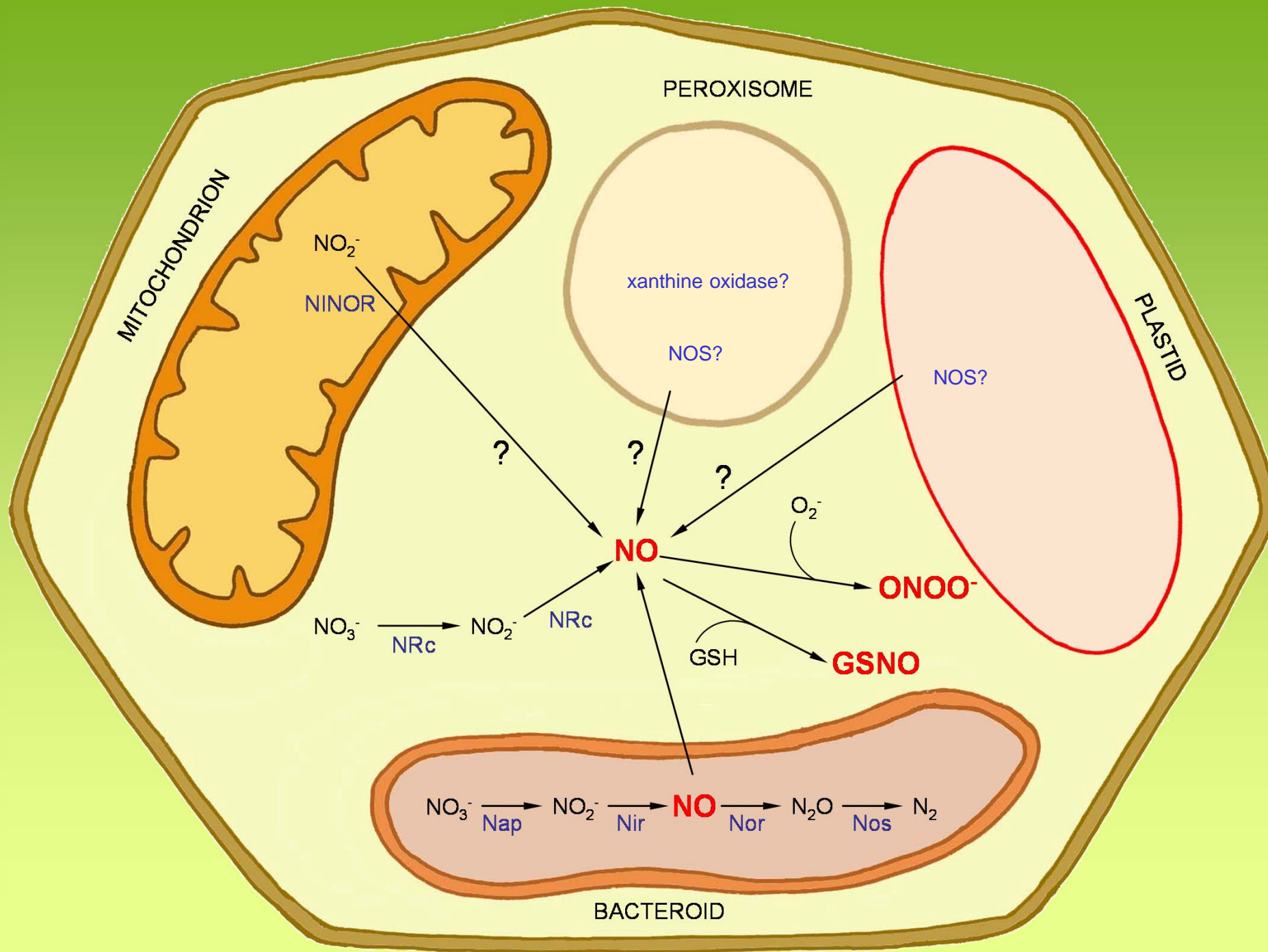
# ROS metabolism in nodules

complex interaction (and balance) of ROS and antioxidants among cell compartments

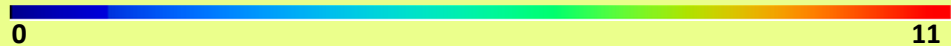


# RNS metabolism in nodules

nitric oxide metabolism and transport are poorly known



Metabolic pathway or enzyme class	Protein	Gene ID (TC/Mt3.5)	Leaf	Petiole	Stem	Veg Bud	Flower	Pod	Root	Nodule	Root 0	Nod 4	Nod 10	Nod 14	Nod 16*	
Ascorbate biosynthesis	GMP	Mt3.5Chr3.3805														
	GalLDH	Mt3.5.1Chr4.4628														
Thiol biosynthesis	γECS	Mt3.5.1Chr5.526c														
	GSHS	Mt3.5.1Chr7.6630														
	hGSHS	Mt3.5.1Chr7.6629														
Peroxide detoxification	Apx	Mt3.5Chr3.5209														
	Apx	Mt3.5Chr3.6657														
	Apx	Mt3.5.1Chr4.3262														
	Apx	Mt3.5.1Chr4.4085														
	Apx	Mt3.5.1Chr5.334														
	Apx	Mt3.5.1Chr8.4124														
	DR	TC142960														
	DR	TC160649														
	MR	TC148060														
	MR	TC144388														
	GR	TC161211														
	GR	TC150698														
	Superoxide detoxification	Catalase	TC151136													
CuZnSOD		Mt3.5.1Chr4.3020														
CuZnSOD		Mt3.5.1Chr7.6665														
CuZnSOD		Mt3.5.1Chr6.1025b														
MnSOD		TC144459														
FeSOD		TC148846														
Thiol peroxidases	Gpx	TC148518														
	Gpx	Mt3.5.1Chr1.724														
	Gpx	Mt3.5.1Chr8.4849														
	Gpx	Mt3.5.1Chr8.4850														
	Gpx	Mt3.5.1Chr8.5356														
	Gpx	Mt3.5.1Chr1.3012														
	2C-Prx	AC146630.25.2a														
	2C-Prx	Mt3.5.1Chr1.5087														
	PrxQ	Mt3.5.1Chr4.7318														
	Protein disulfide reductases	Trx/Grx	Mt3.5.1Chr1.2406													
Trx/Grx		Mt3.5.1Chr1.4644														
Trx/Grx		Mt3.5.1Chr1.5411														
Trx/Grx		Mt3.5Chr3.5347														
Trx/Grx		Mt3.5Chr3.6854														
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Trx/Grx		Mt3.5.1Chr4.4715a														
Trx/Grx		Mt3.5.1Chr4.749														
Trx/Grx		Mt3.5.1Chr5.1359a														
Trx/Grx		Mt3.5.1Chr5.1991														
Trx/Grx		Mt3.5.1Chr5.3294														
Trx/Grx		Mt3.5.1Chr7.311														
Trx/Grx		Mt3.5.1Chr7.4199														
Trx/Grx		Mt3.5.1Chr7.5195														
Trx/Grx		Mt3.5.1Chr7.5472b														
Glutathione S-transferases		Trx/Grx	Mt3.5.1Chr7.6129													
		Trx/Grx	Mt3.5.1Chr7.770e													
	Trx/Grx	Mt3.5.1Chr8.3560														
	GST	Mt3.5.1Chr1.5511														
	GST	Mt3.5.1Chr2.4037														
	GST	Mt3.5.1Chr4.4924														
	GST	Mt3.5.1Chr5.3239														
	GST	Mt3.5.1Chr5.3544														
	GST	Mt3.5.1Chr5.6793														
	GST	Mt3.5.1Chr5.8195														
	GST	Mt3.5.1Chr7.3104														
	GST	Mt3.5.1Chr7.3107														
	GST	Mt3.5.1Chr7.3108														
	GST	Mt3.5.1Chr7.3141														
	GST	Mt3.5.1Chr7.3144														
	GST	Mt3.5.1Chr7.3145														
	GST	Mt3.5.1Chr7.3147														
	Metal homeostasis and detoxification	GST	Mt3.5.1Chr7.3149													
		GST	Mt3.5.1Chr7.3155													
GST		Mt3.5.1Chr7.5757														
GST		Mt3.5.1Chr8.2226														
GST		Mt3.5.1Chr8.4851														
MT	Mt3.5.1Chr4.1021															
MT	Mt3.5.1Chr8.2470															
NAS	Mt3.5.1Chr1.3615															
NAS	Mt3.5.1Chr2.2244															



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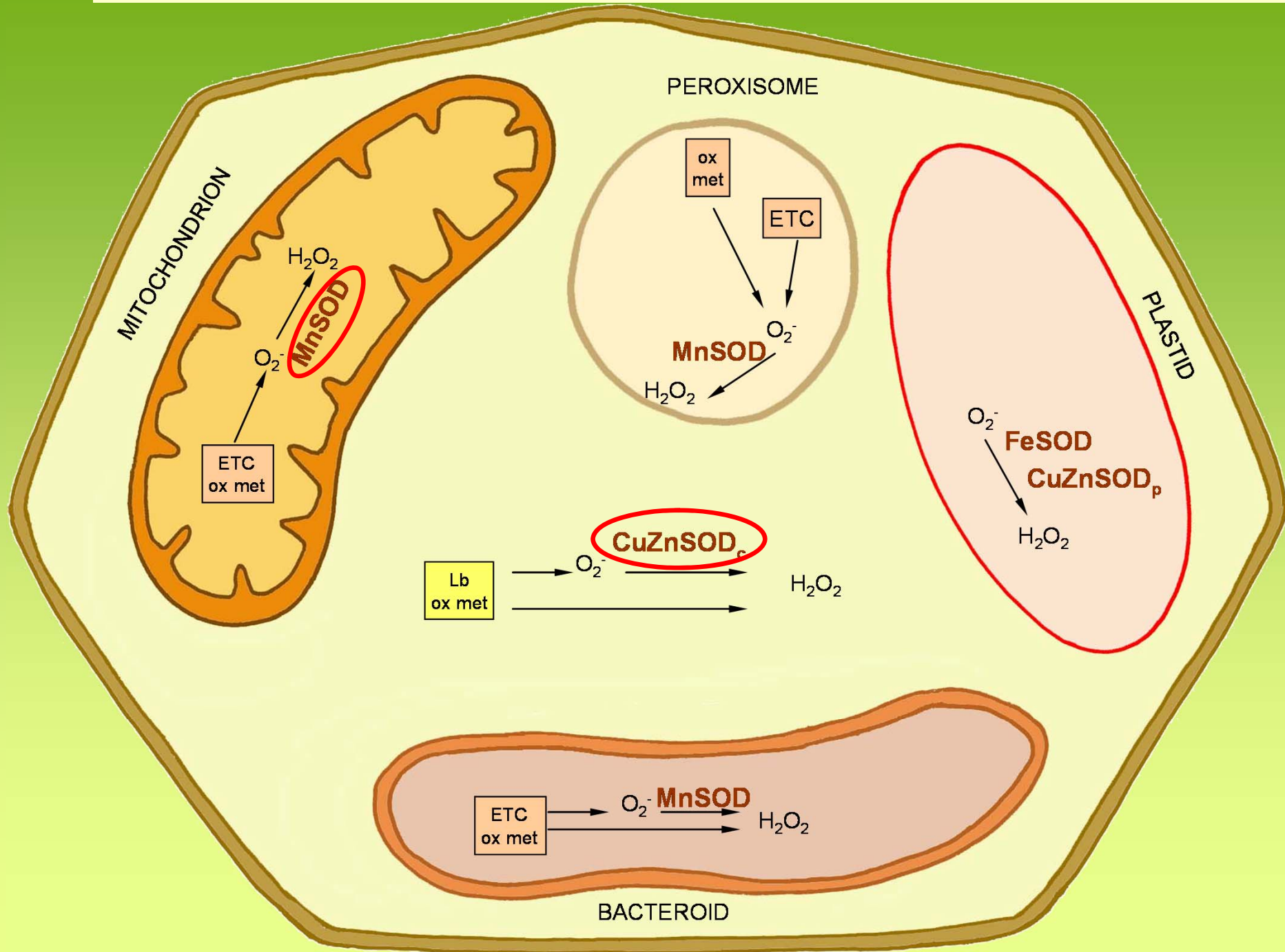


## Four aspects of ROS/RNS metabolism in nodules

- **Superoxide dismutases:** ROS metabolism
- **Thiols (glutathione and homoglutathione synthesis):** ROS/RNS metabolism
- **Nitrosoglutathione reductase:** RNS metabolism
- **Nonsymbiotic and truncated hemoglobins:** ROS/RNS interaction/metabolism

# Superoxide dismutases in nodules

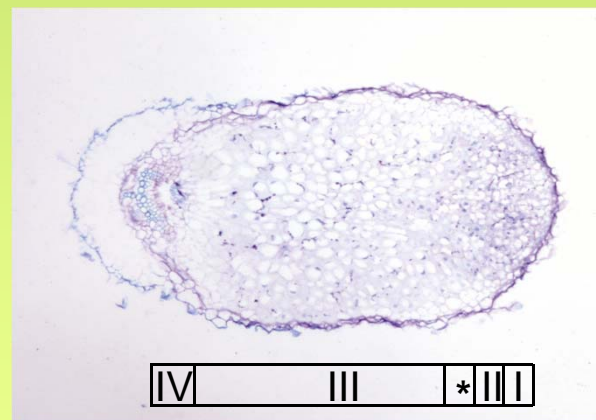
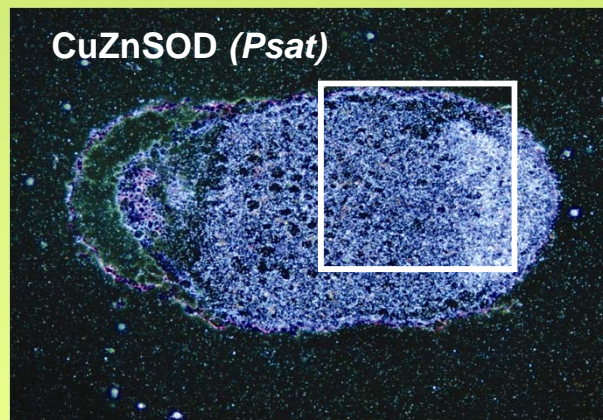
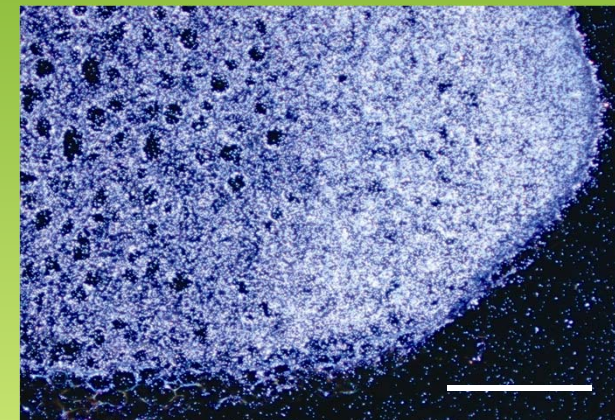
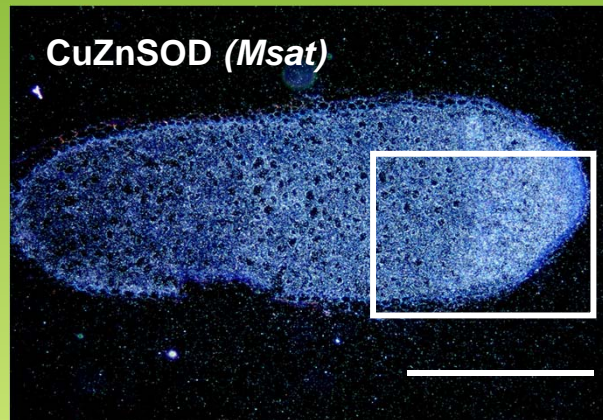
different isoforms are present in each cellular compartment





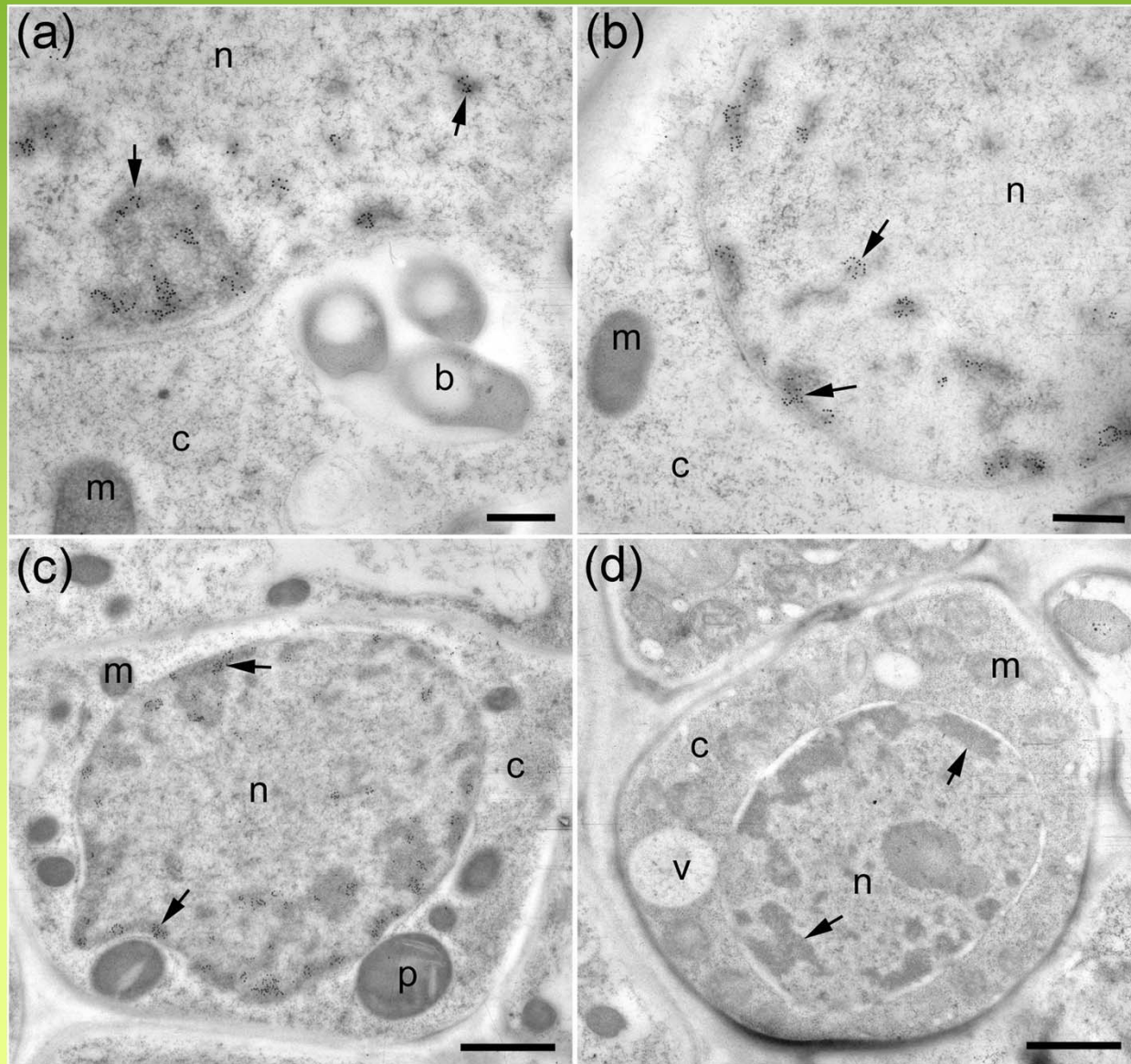
# Cytosolic CuZnSOD: *in situ* RNA localization in indeterminate nodules

expression is mainly localized in zones I + II



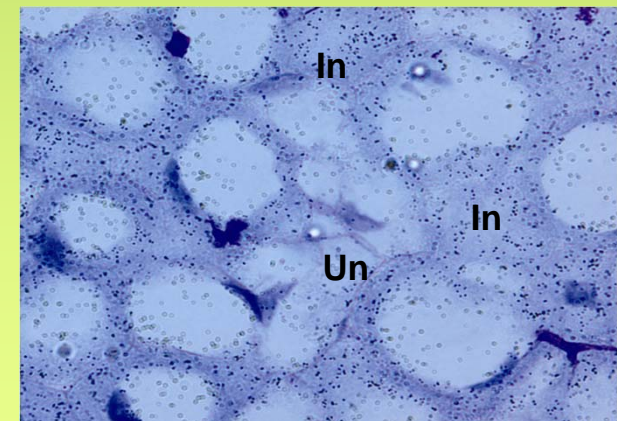
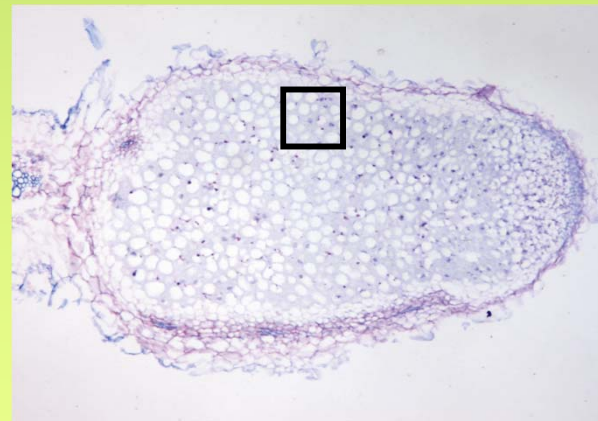
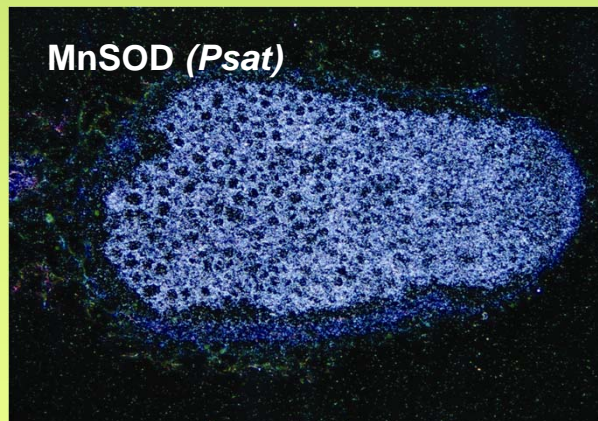
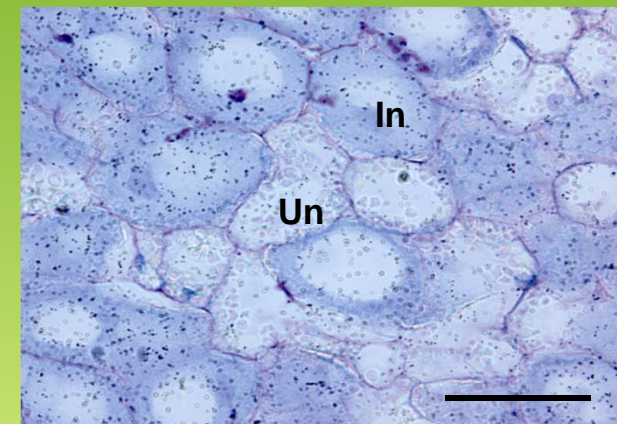
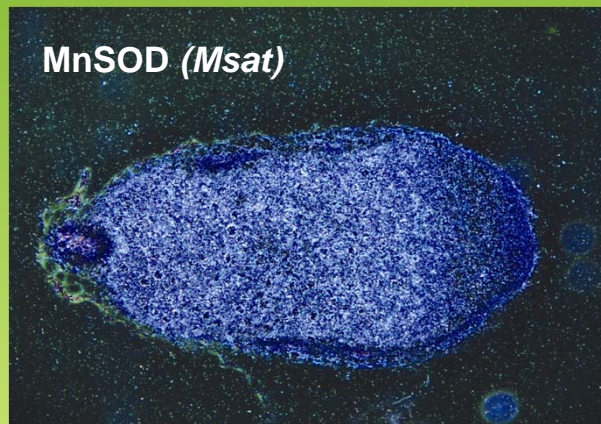
# Cytosolic CuZnSOD: immunolocalization of the protein in *Sesbania* nodules

the 'cytosolic' enzyme is present in the nucleus in addition to the cytosol of stem nodules



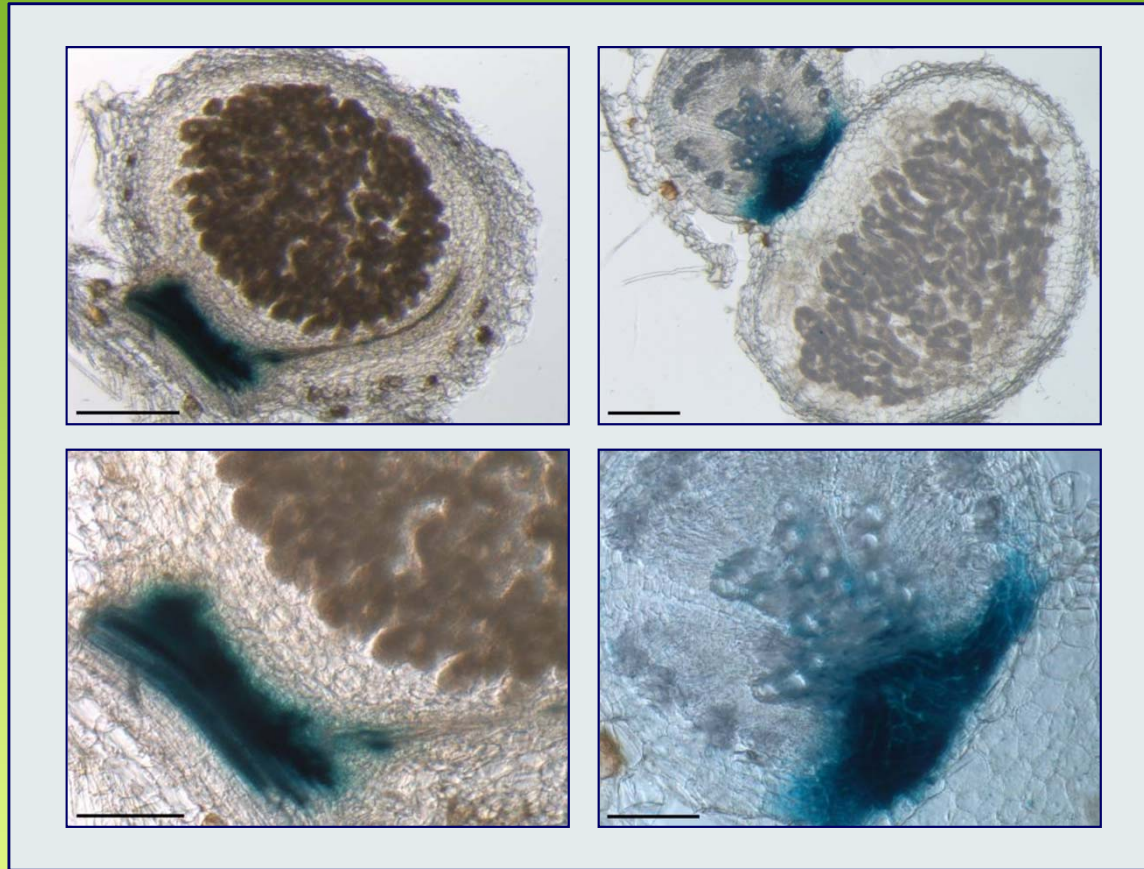
# Mitochondrial MnSOD: *in situ* RNA localization in indeterminate nodules

expression is mainly localized in the infected zone



# Mitochondrial MnSOD: localization of promoter activity in *Lotus japonicus*

promoter activity can be detected in vascular bundles



↑  
Mature nodules (34-37 dpi)

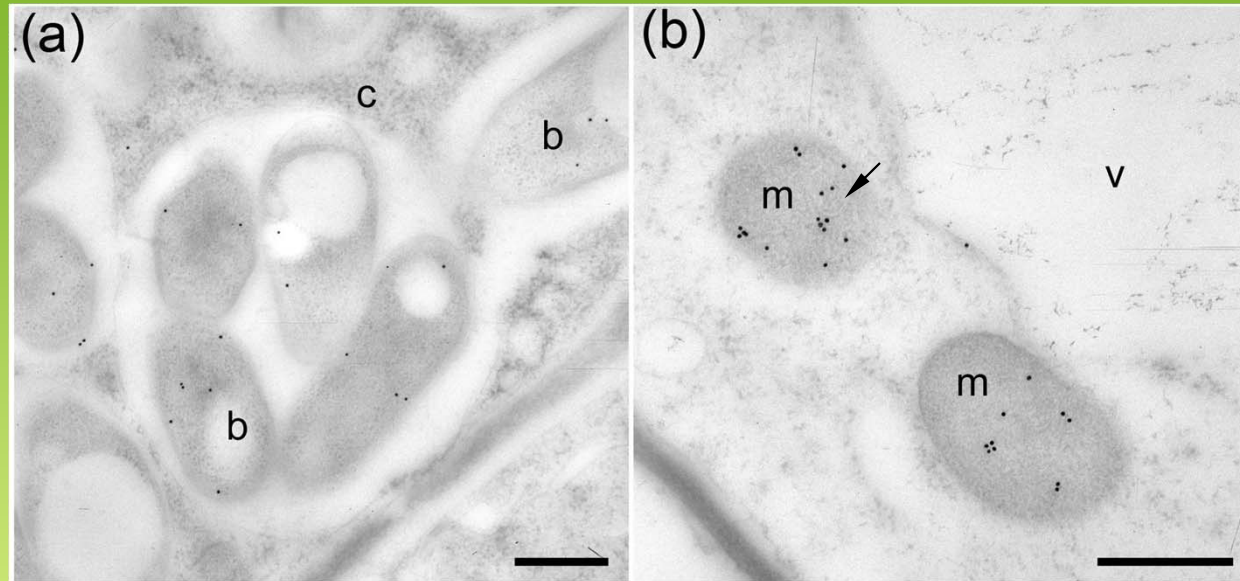
Old nodules (50-56 dpi) →



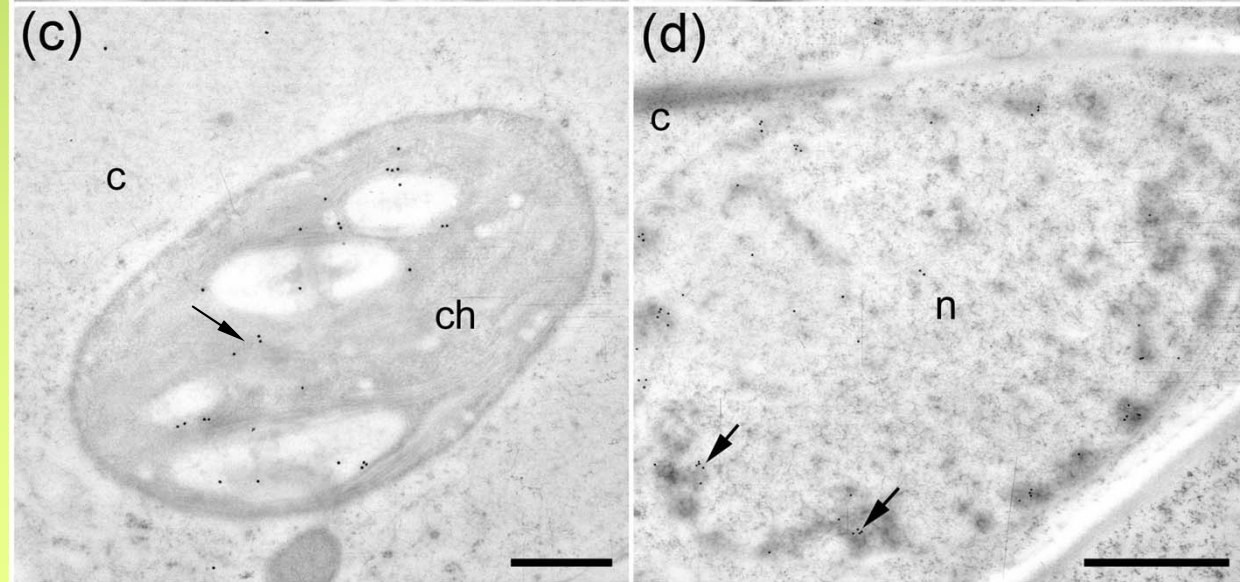
# MnSOD and FeSOD: immunolocalization of the proteins in *Sesbania* nodules

MnSOD is present in the mitochondria of vascular bundle cells and FeSOD in the chloroplasts of stem nodules and in nuclei of stem and root nodules

MnSOD

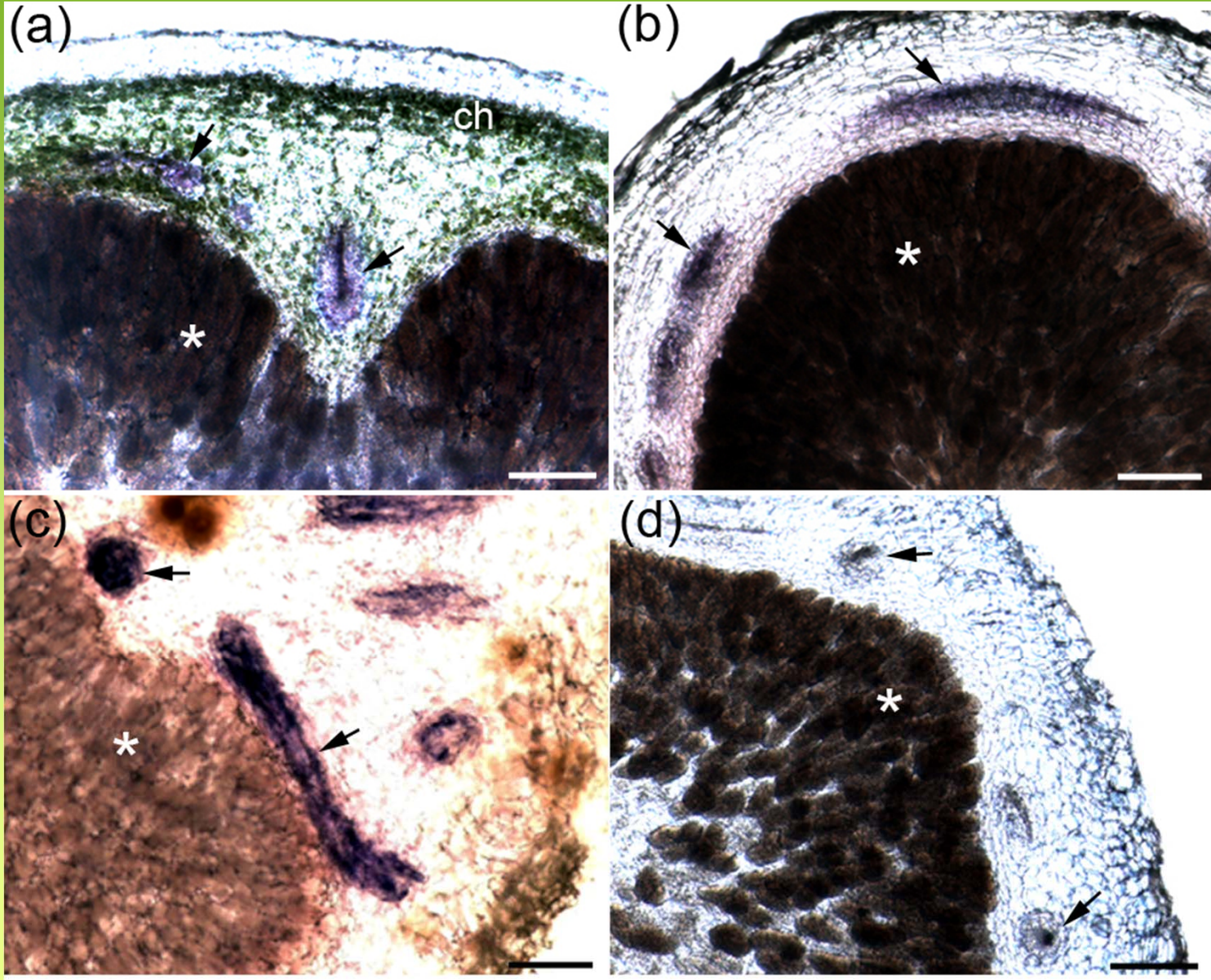


FeSOD



## Superoxide production in *Sesbania* nodules

this ROS is mainly produced in the vascular bundles in the cortex of stem and root nodules





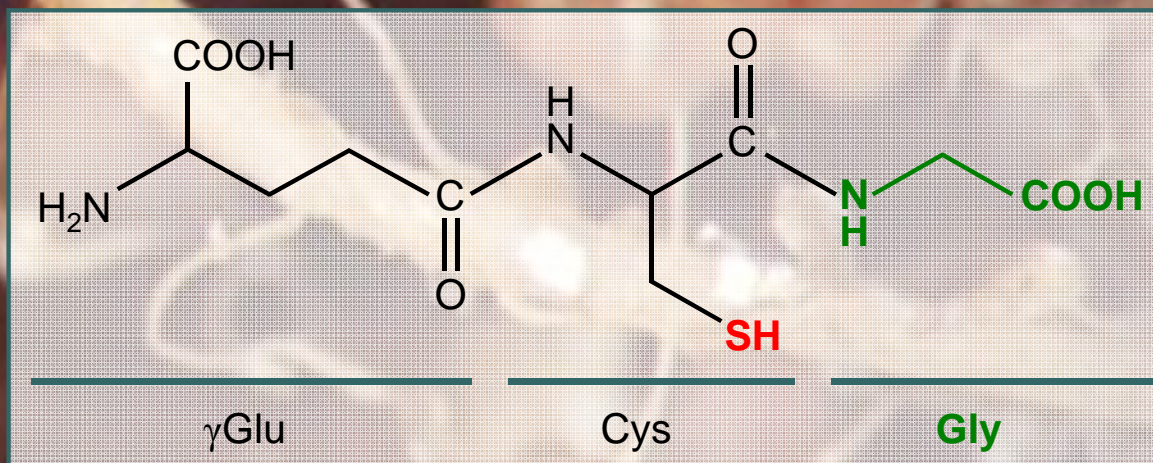
## Four aspects of ROS/RNS metabolism in nodules

- **Superoxide dismutases: ROS metabolism**
- **Thiols (glutathione and homoglutathione synthesis): ROS/RNS metabolism**
- **Nitrosoglutathione reductase: RNS metabolism**
- **Nonsymbiotic and truncated hemoglobins: ROS/RNS interaction/metabolism**

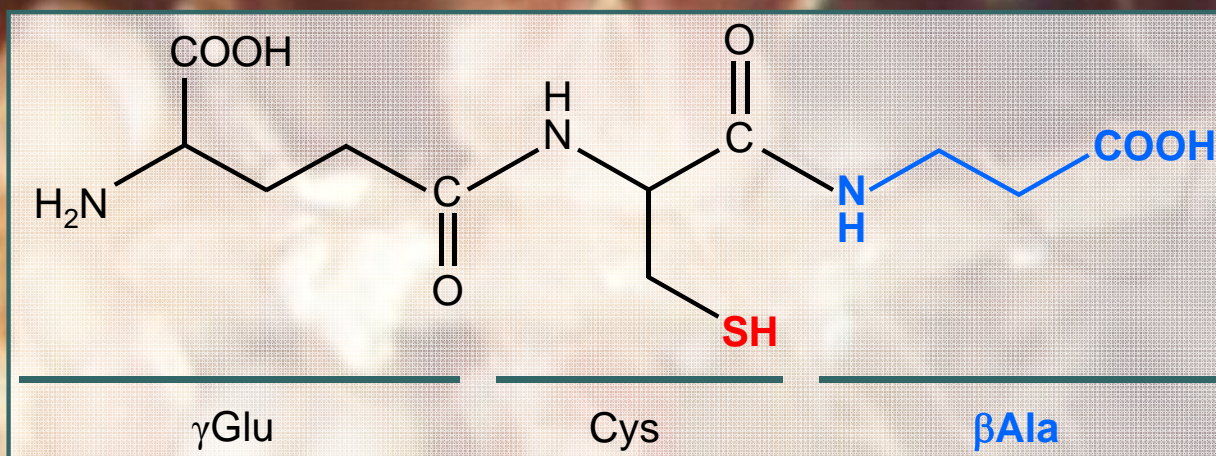
## Thiol compounds

GSH is widespread in organisms but hGSH is exclusive of legumes

**Glutathione  
(GSH)**



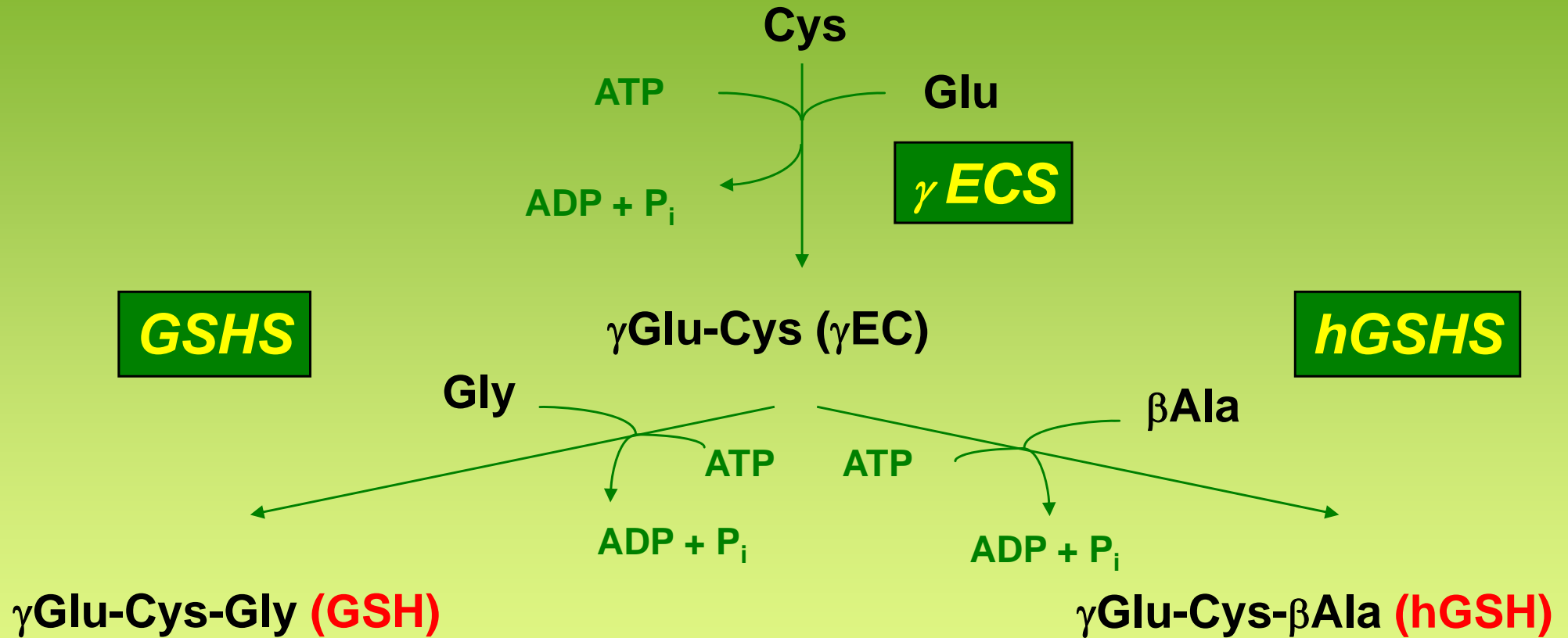
**Homoglutathione  
(hGSH)**





# Thiol biosynthesis

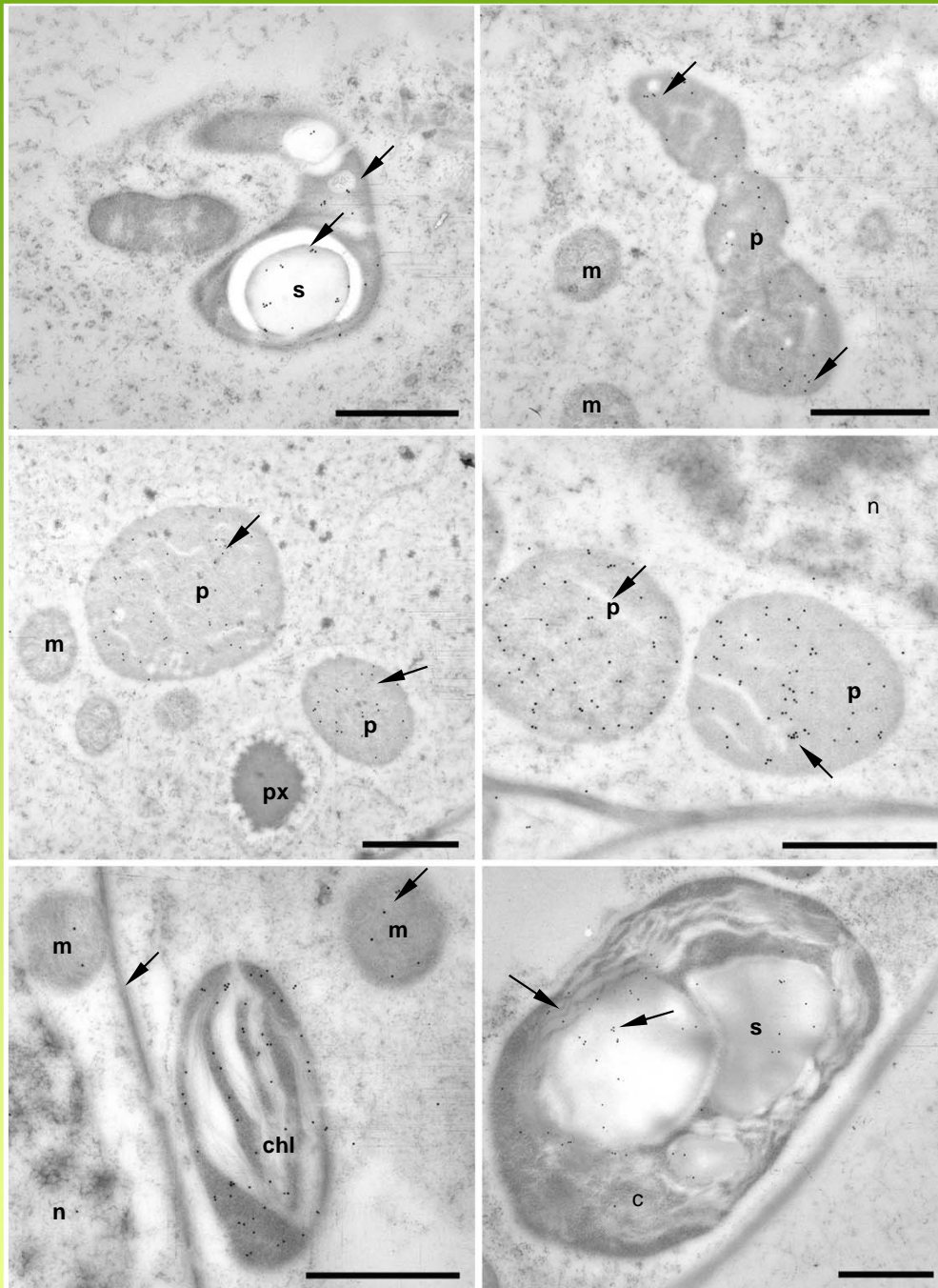
GSH and hGSH synthesis involves two sequential ATP-dependent reactions



$\gamma$ ECS:  $\gamma$ -glutamylcysteine synthetase  
(h)GSHS: (homo)glutathione synthetases

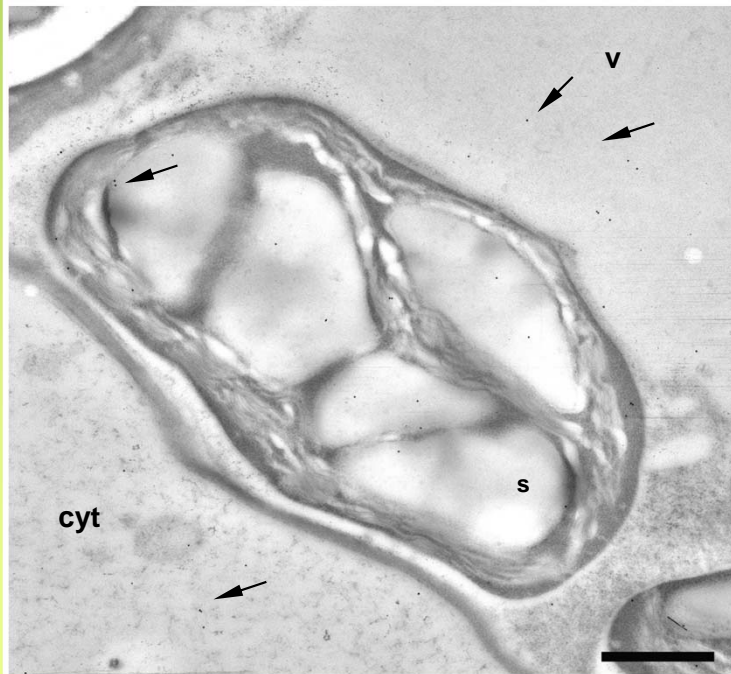
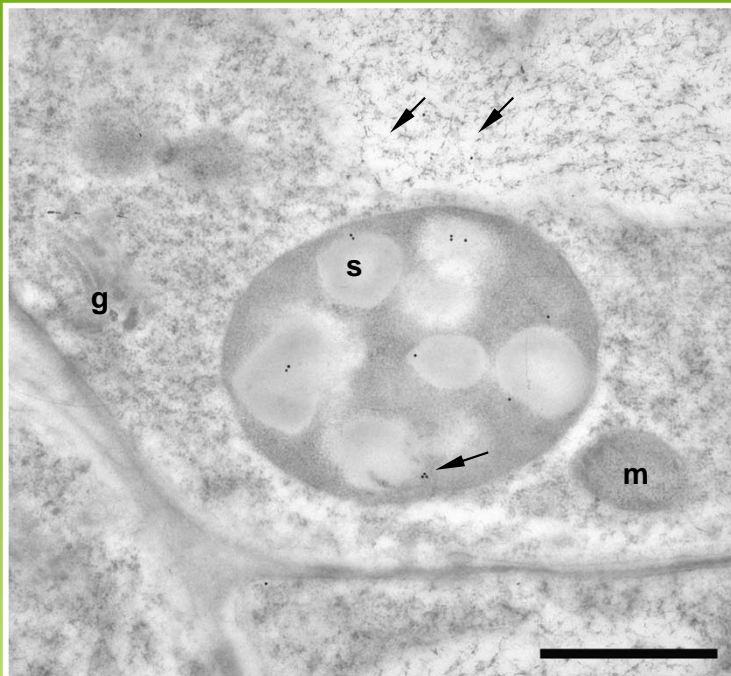
## Localization of thiol biosynthesis

$\gamma$ ECS is localized exclusively in  
the plastids of nodules and in  
the chloroplasts of leaves and  
stem nodules (*Sesbania  
rostrata*)



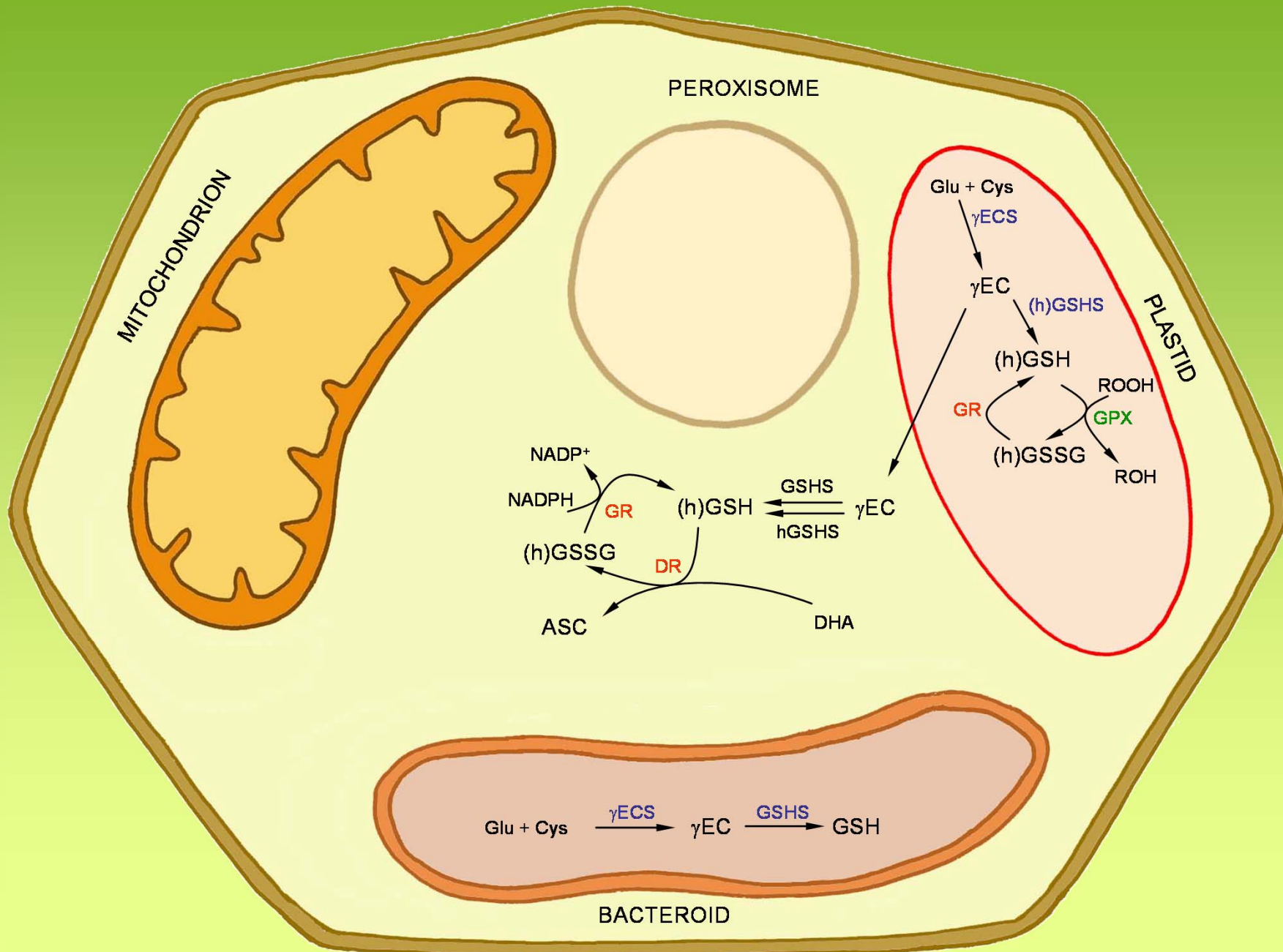
## Localization of thiol biosynthesis

GSHS and hGSHS are localized in both the plastids and cytosol in roots, nodules and leaves



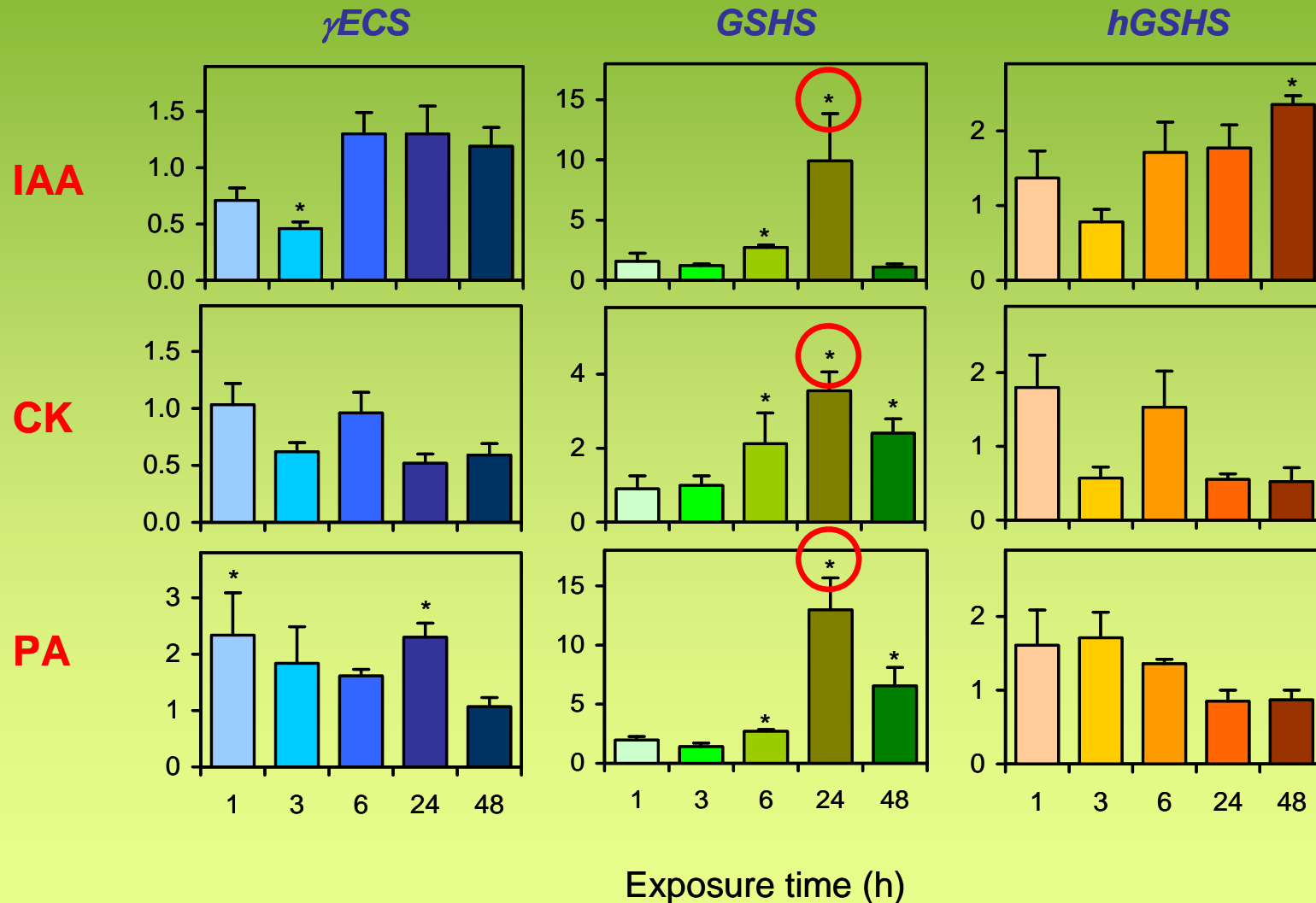
## Thiol metabolism in nodules

- the enzymes  $\gamma$ ECS and (h)GSHS are present in the plastids and/or cytosol
- (h)GSH synthesis in the cytosol requires export of  $\gamma$ EC from plastids; this has regulatory implications
- bacteroids have their own  $\gamma$ ECS and GSHS, which have poor homology with the plant enzymes



# Thiol metabolism

expression of the *GS* gene, but not of the *hGS* gene, is induced by indole-3-acetic acid (IAA), cytokinins (CK), and polyamines (PA), suggesting different functions for the corresponding enzymes (GS and hGS) and thiols (GSH and hGS)



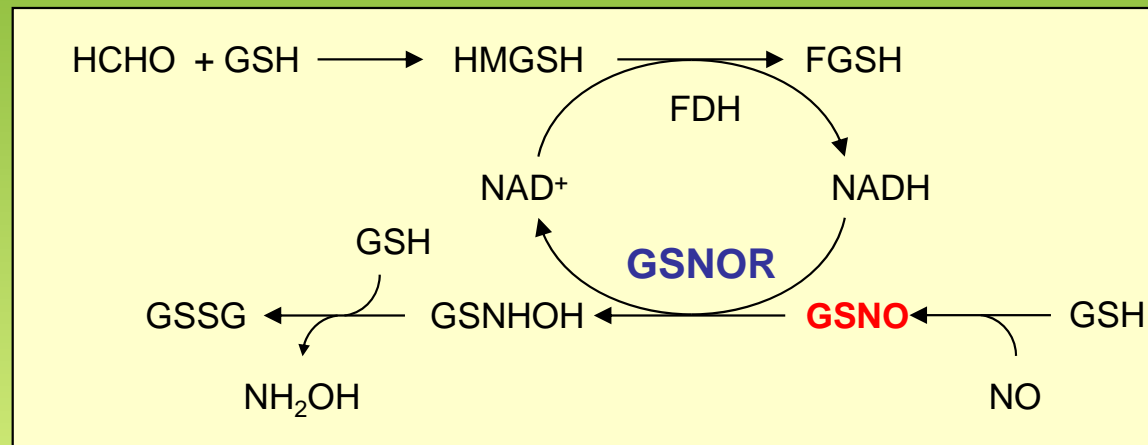


## Four aspects of ROS/RNS metabolism in nodules

- **Superoxide dismutases: ROS metabolism**
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## Nitrosoglutathione (**GSNO**) and nitrosoglutathione reductase (**GSNOR**)

- **GSNO** is not just a NO reservoir or a form for NO transport. In animals, GSNO participates in the post-translational modification of proteins by nitrosylation
- In plants, **GSNO** is involved in abiotic and biotic stress responses.



HMGS: hydroxymethyl-GSH      FGS: formyl-GSH

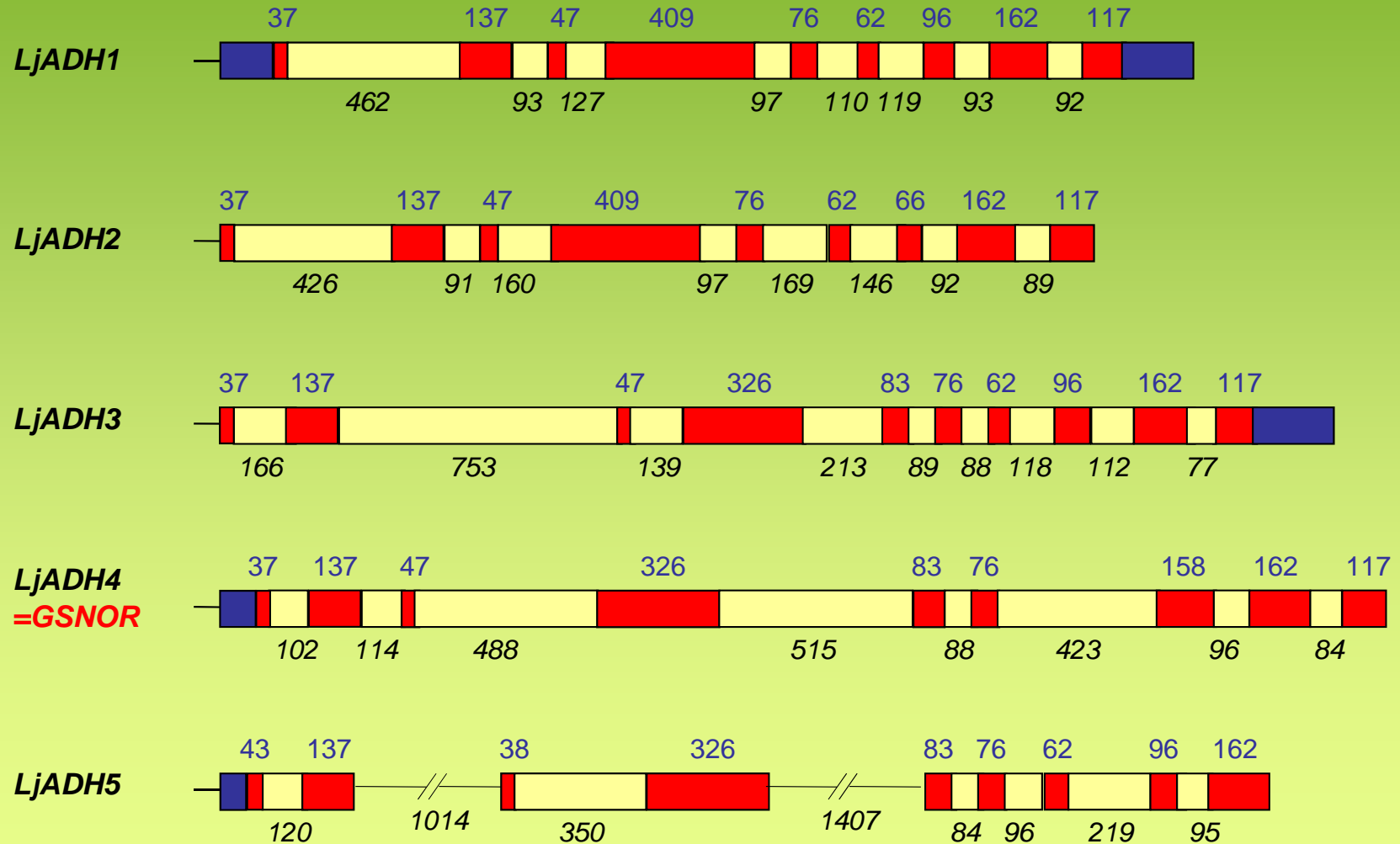


Animal GSNOR (ADH3)

- **GSNOR** belongs to the alcohol dehydrogenase family of enzymes and has a dual function: GSNO catabolizing activity (**GSNOR**) and GSH-dependent formaldehyde dehydrogenase activity (**FDH**)
- In animals, and presumably also in plants, **GSNOR** activity catalyzes the reduction of GSNO by NADH producing GSSG and  $\text{NH}_2\text{OH}$  and regulates NO levels
- In plants, **GSNOR** is involved in abiotic and biotic stress (such as heavy metals, wounding, and pathogen attack) and in response to stress-related compounds (jasmonic and salicylic acids). In leaves, GSNOR has been localized in the chlorenchyma cells

# Alcohol dehydrogenase/GSNOR gene family of *Lotus japonicus*

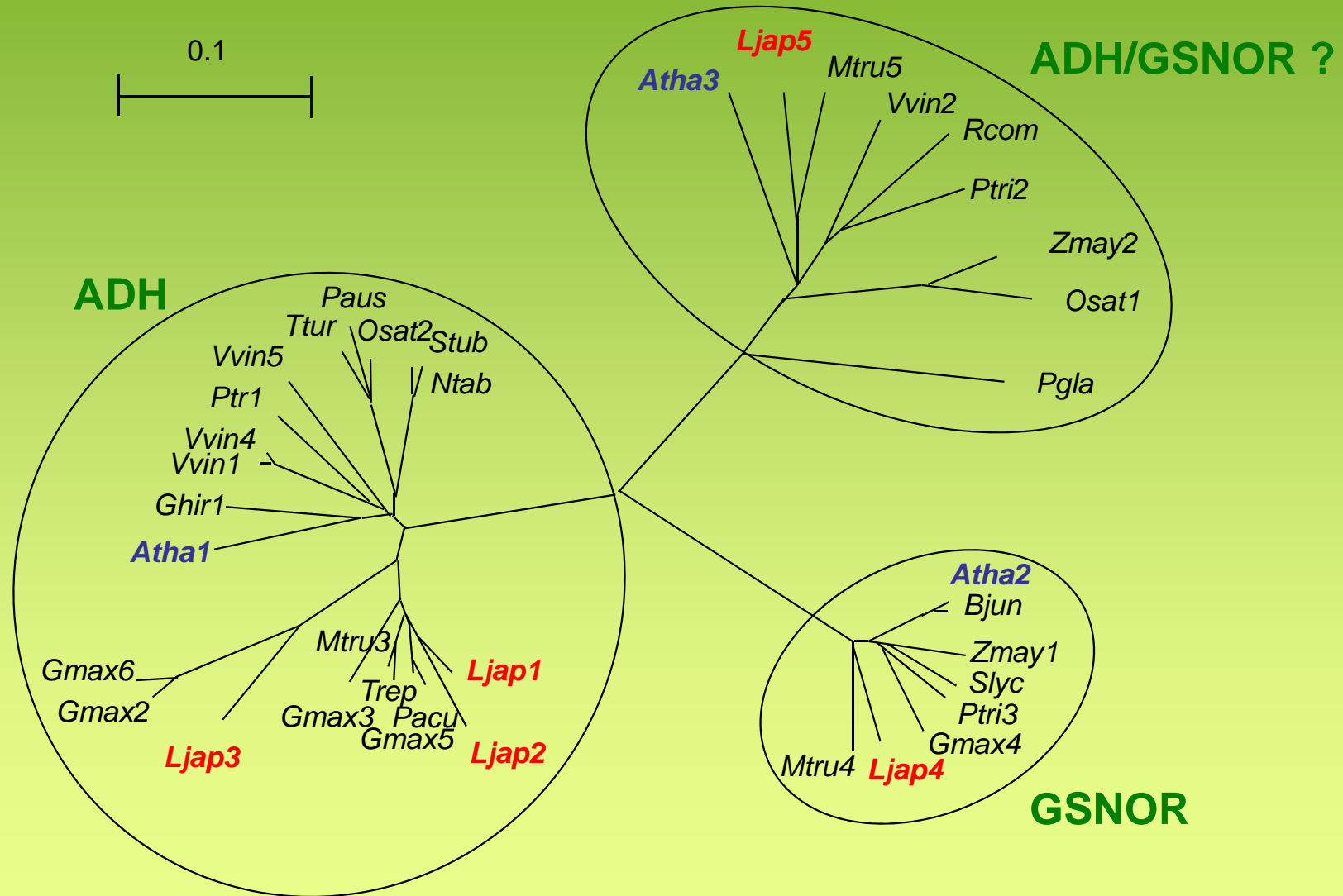
at least five genes have been identified in this model legume; one of them, which we have designated as *LjADH4*, is orthologous to the *Arabidopsis* *GSNOR*





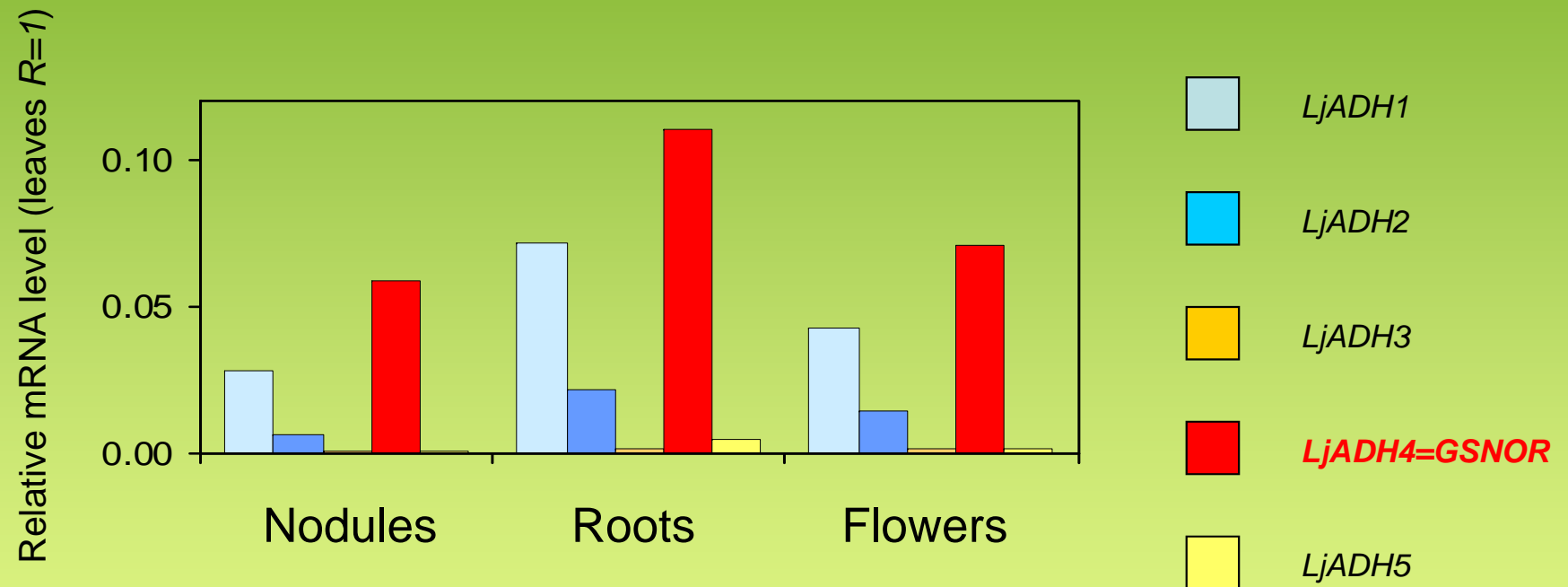
# Alcohol dehydrogenase/GSNOR enzyme family: phylogenetic analysis of higher plant proteins

predicted proteins group in three clusters: one includes typical ADHs, one includes GSNOR enzymes, and one has intermediate sequence homologies and has unknown enzyme activities



## *LjADH/GSNOR* genes: differential expression in plant tissues

*LjGSNOR* (and other genes of the alcohol dehydrogenase family) is expressed in all plant organs, including nodules, although at lower levels than in leaves



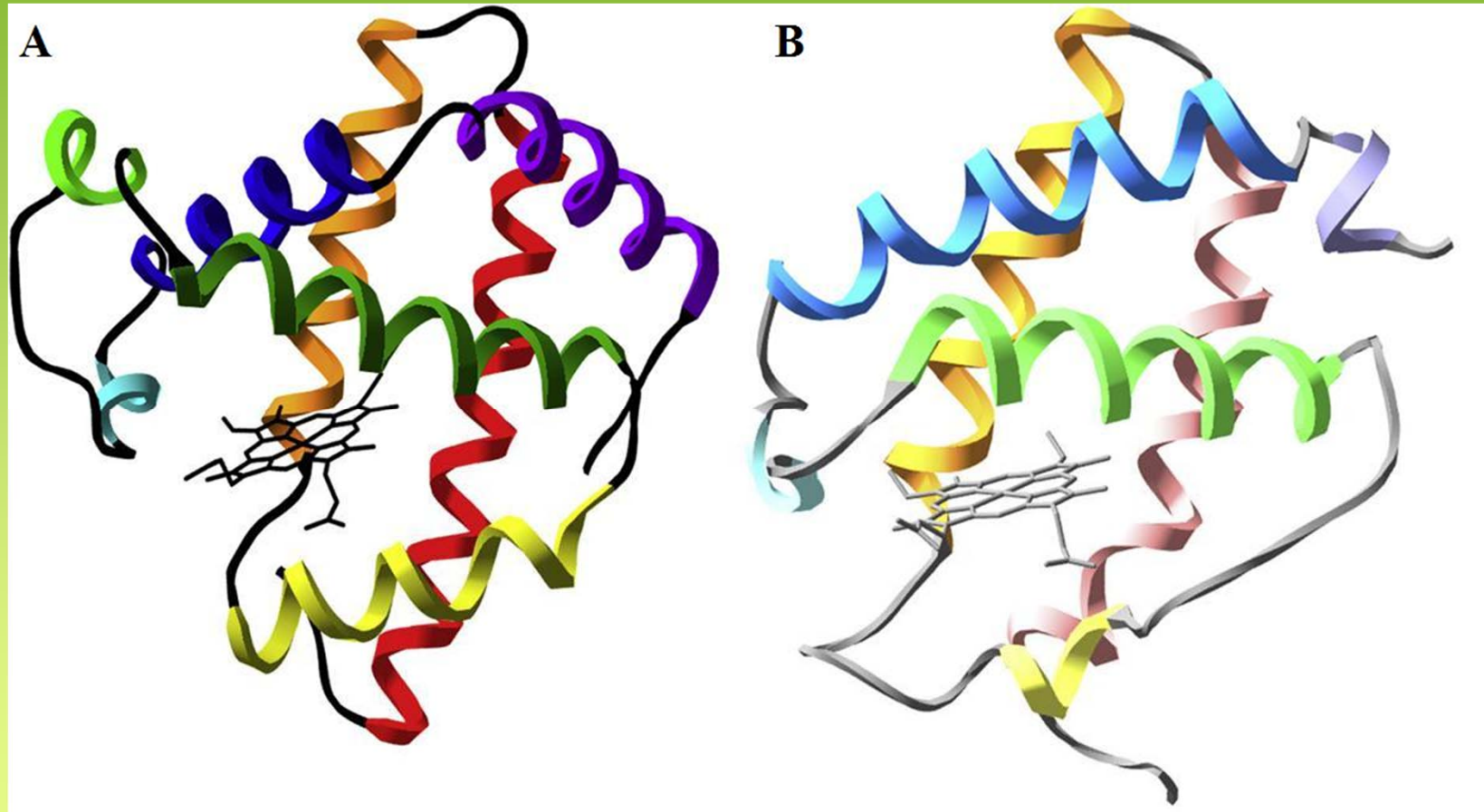


## Four aspects of ROS/RNS metabolism in nodules

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# Plant hemoglobins

- three types: symbiotic (leghemoglobins and some hemoglobins from actinorhizal plants), nonsymbiotic, and truncated
- nonsymbiotic are further divided into class 1 and class 2, based on amino acid sequences and biochemical properties



**(A)** Classical “3-on-3” structure of leghemoglobins and nonsymbiotic hemoglobins

**(B)** “2-on-2” structure of truncated hemoglobins

[Hoy & Hargrove (2008) *Plant Physiol Biochem* 46: 371-379]

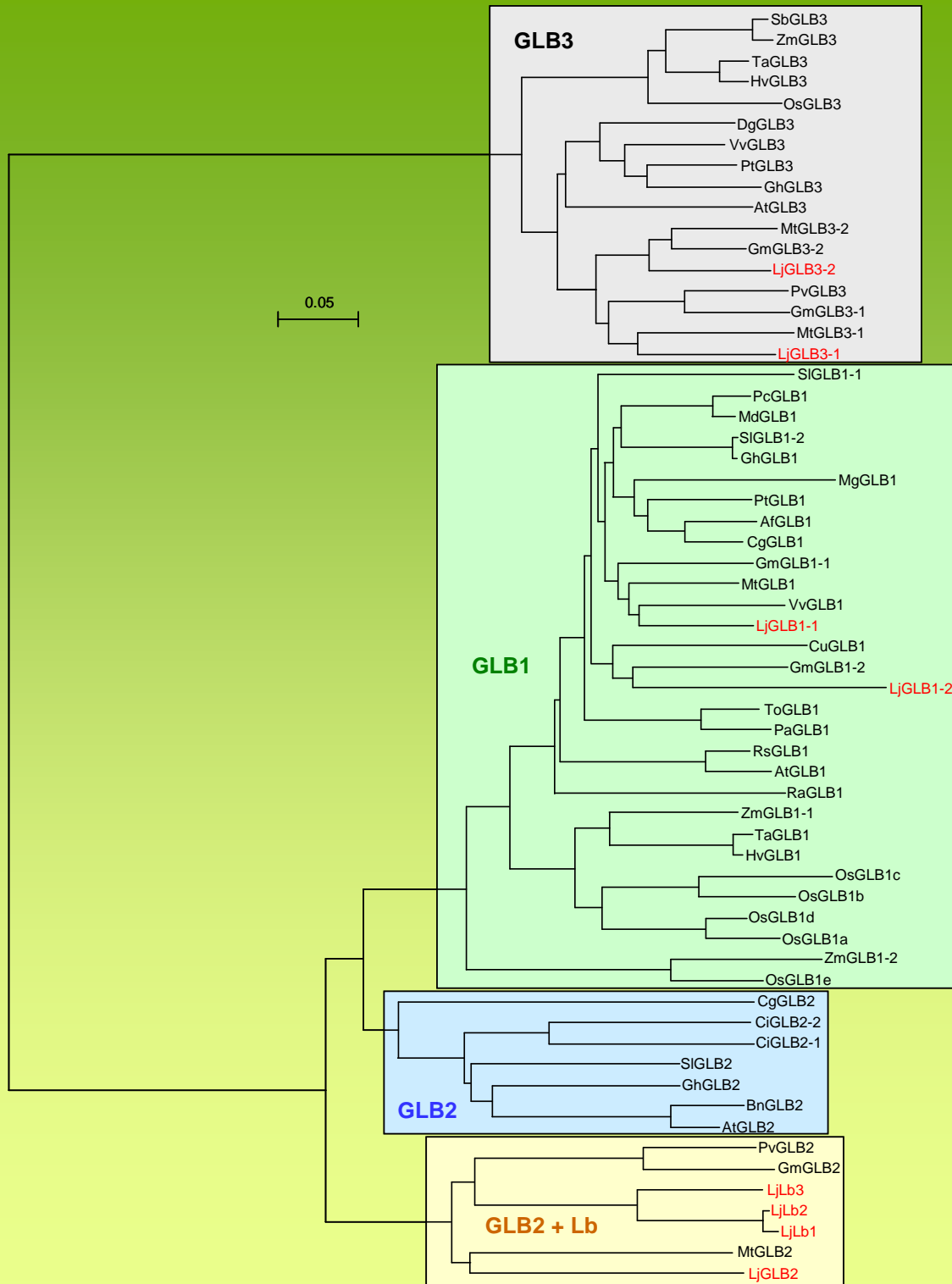
## Nonsymbiotic and truncated hemoglobins of *Lotus japonicus*

- five hemoglobin genes, encoding two class 1 (GLB1), one class 2 (GLB2), and two truncated (GLB3) proteins
- all genes have four exons and three introns, organized similarly to the *GLB1*, *GLB2*, and *GLB3* genes of *Arabidopsis*, respectively



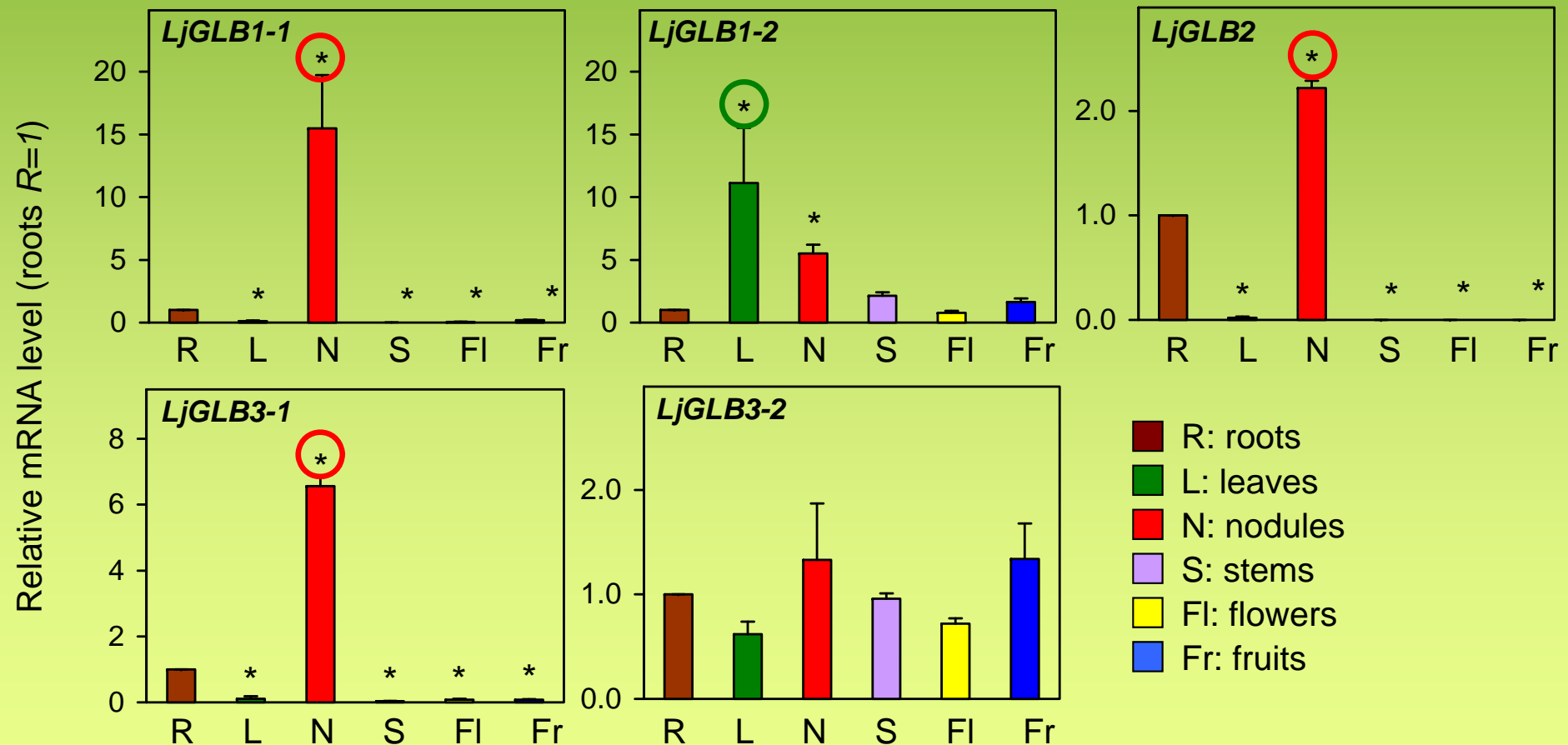
# Phylogenetic analysis of GLB proteins of higher plants

- three clusters corresponding to the three types of hemoglobins
- GLB2s are closely related to leghemoglobins



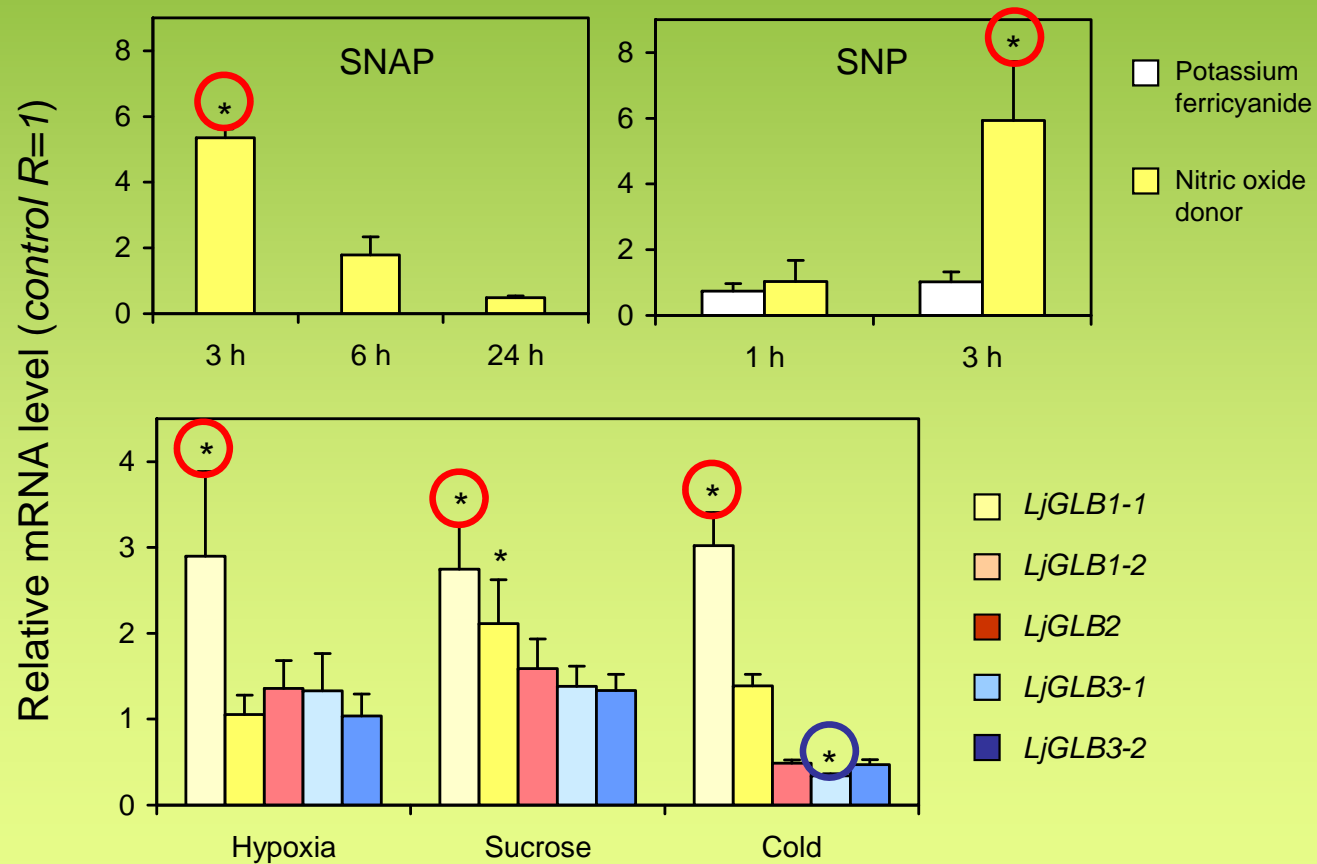
## Hemoglobins of *Lotus japonicus*: differential expression in plant tissues

- one gene of each hemoglobin type (*LjGLB1-1*, *LjGLB2*, and *LjGLB3-1*) is highly expressed in nodules
- *LjGLB1-2* is expressed almost exclusively in leaves and nodules, whereas *LjGLB3-2* is uniformly expressed in all plant organs



## Hemoglobin genes of *Lotus japonicus*: effects of nitric oxide and abiotic stress

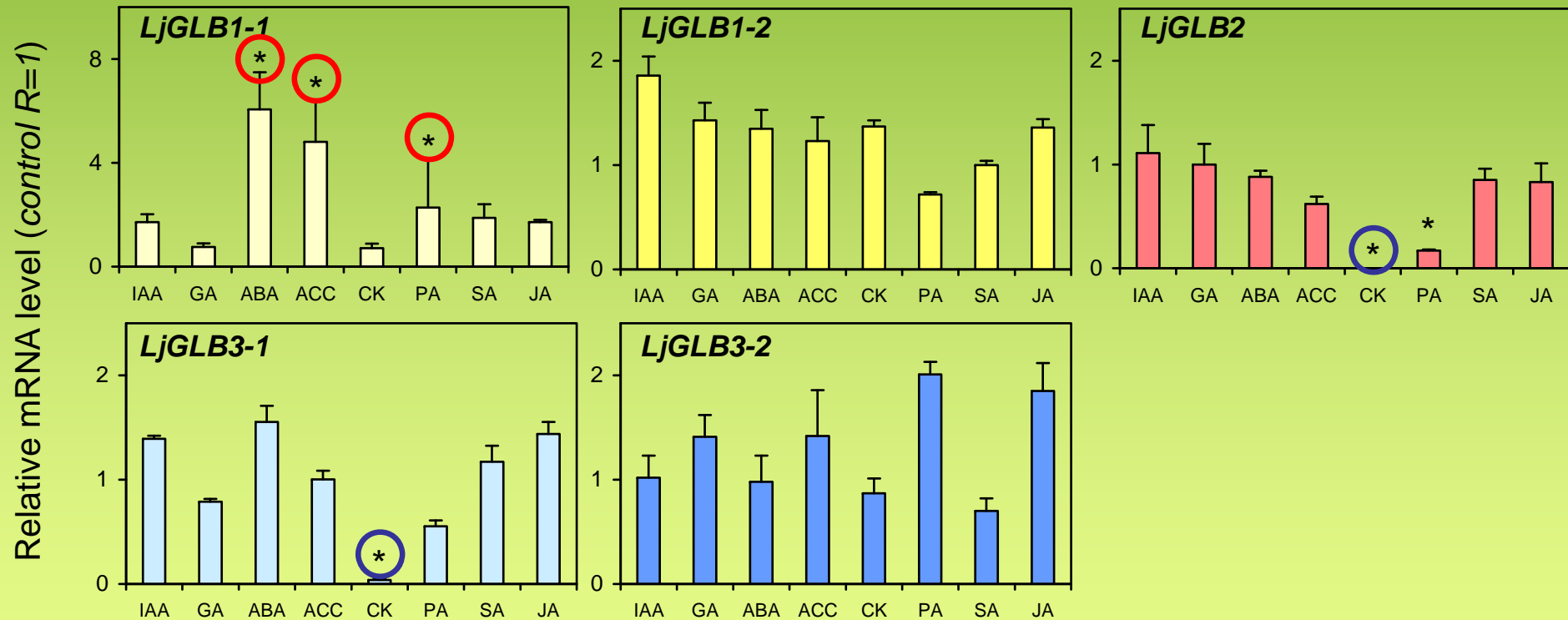
- *LjGLB1-1* was the only gene responsive to NO (using SNAP and SNP as NO donors)
- *LjGLB1-1* was also induced by hypoxia and sucrose, whereas *LjGLB3-1* was induced by low temperatures





## Hemoglobins of *Lotus japonicus*: differential expression in response to hormones

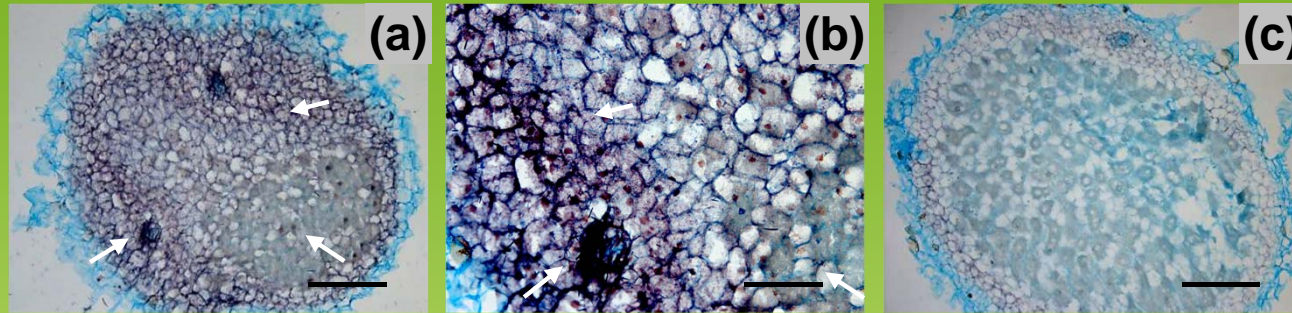
- abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), and polyamines (PA) induce *LjGLB1-1* expression
- cytokinins (CK) suppress *LjGLB2* and *LjGLB3-1* expression



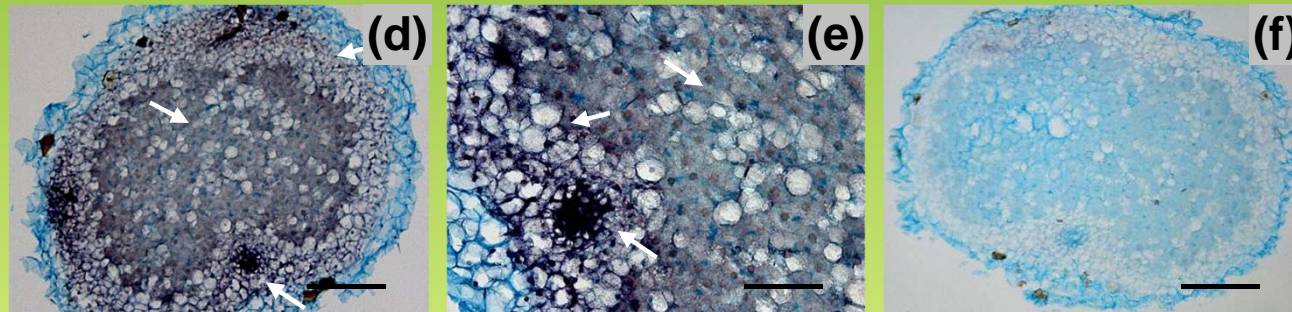
## Hemoglobins of *Lotus japonicus*: *in situ* localization of mRNAs

- *LjGLB1-1* and *LjGLB1-2* mRNAs are localized in the infected zone, vascular bundles, and inner cortex
- *LjGLB2* and *LjGLB3-2* are preferentially expressed in the mid/inner cortex and vascular bundles

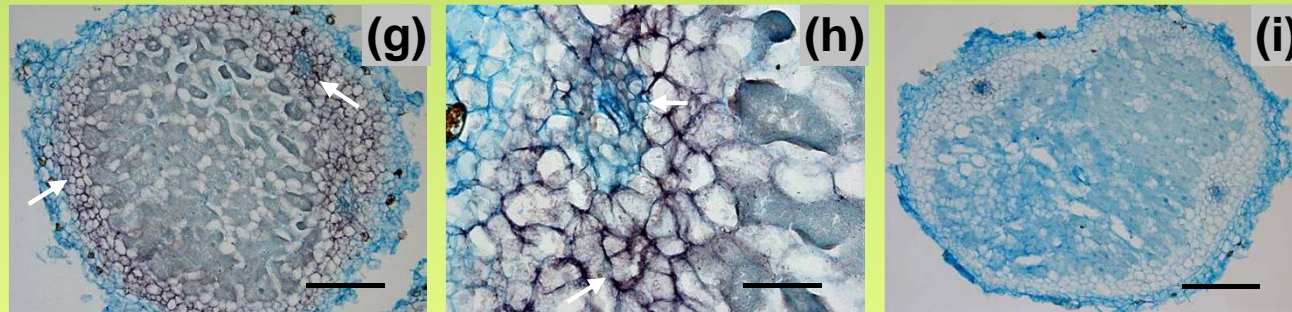
*LjGLB1-1*



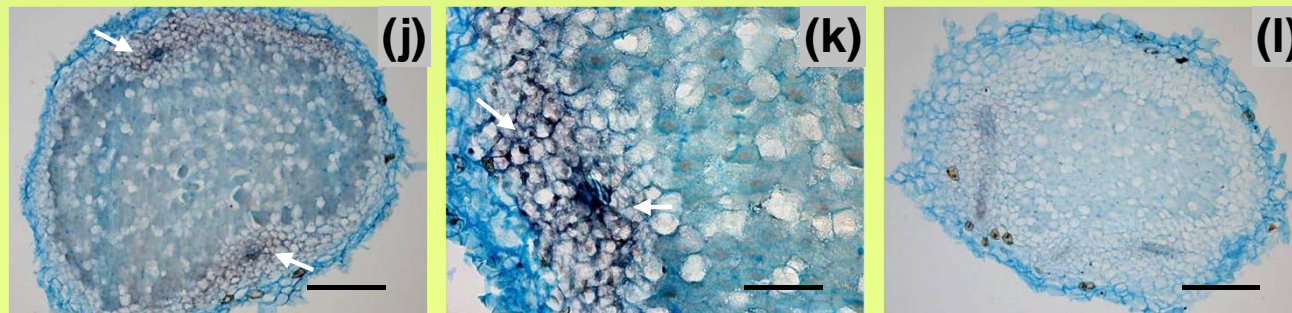
*LjGLB1-2*



*LjGLB2*

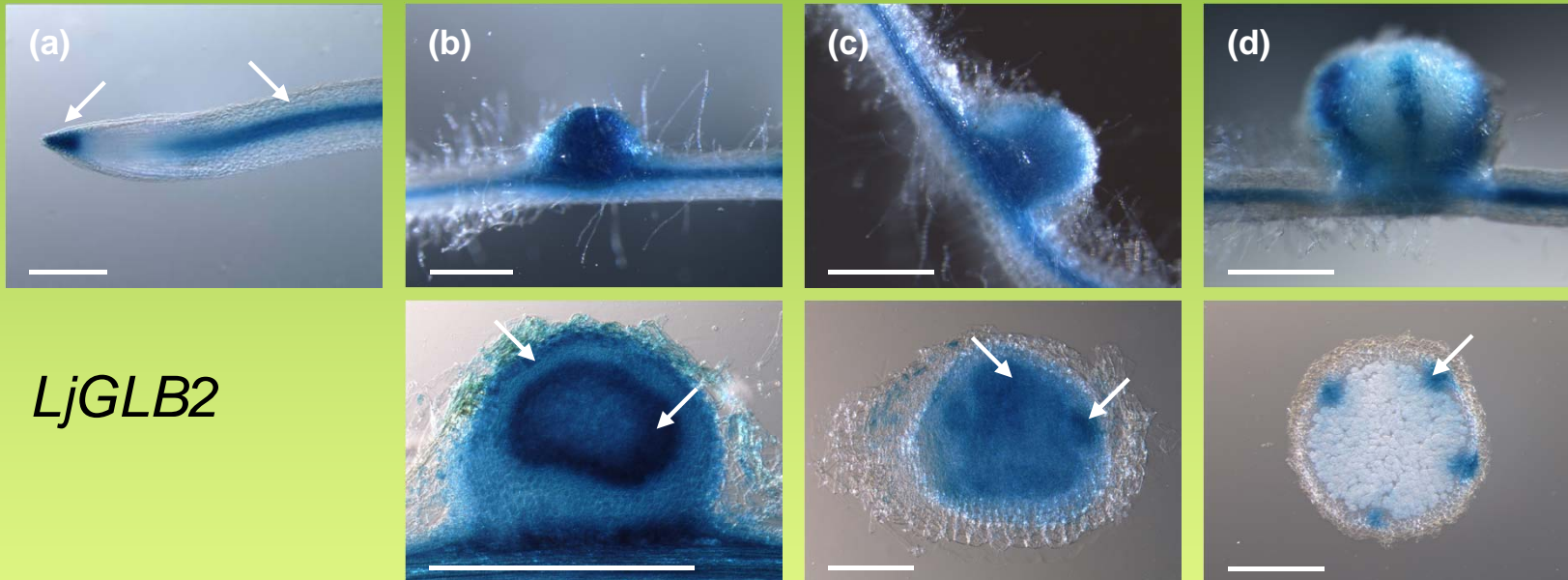


*LjGLB3-2*



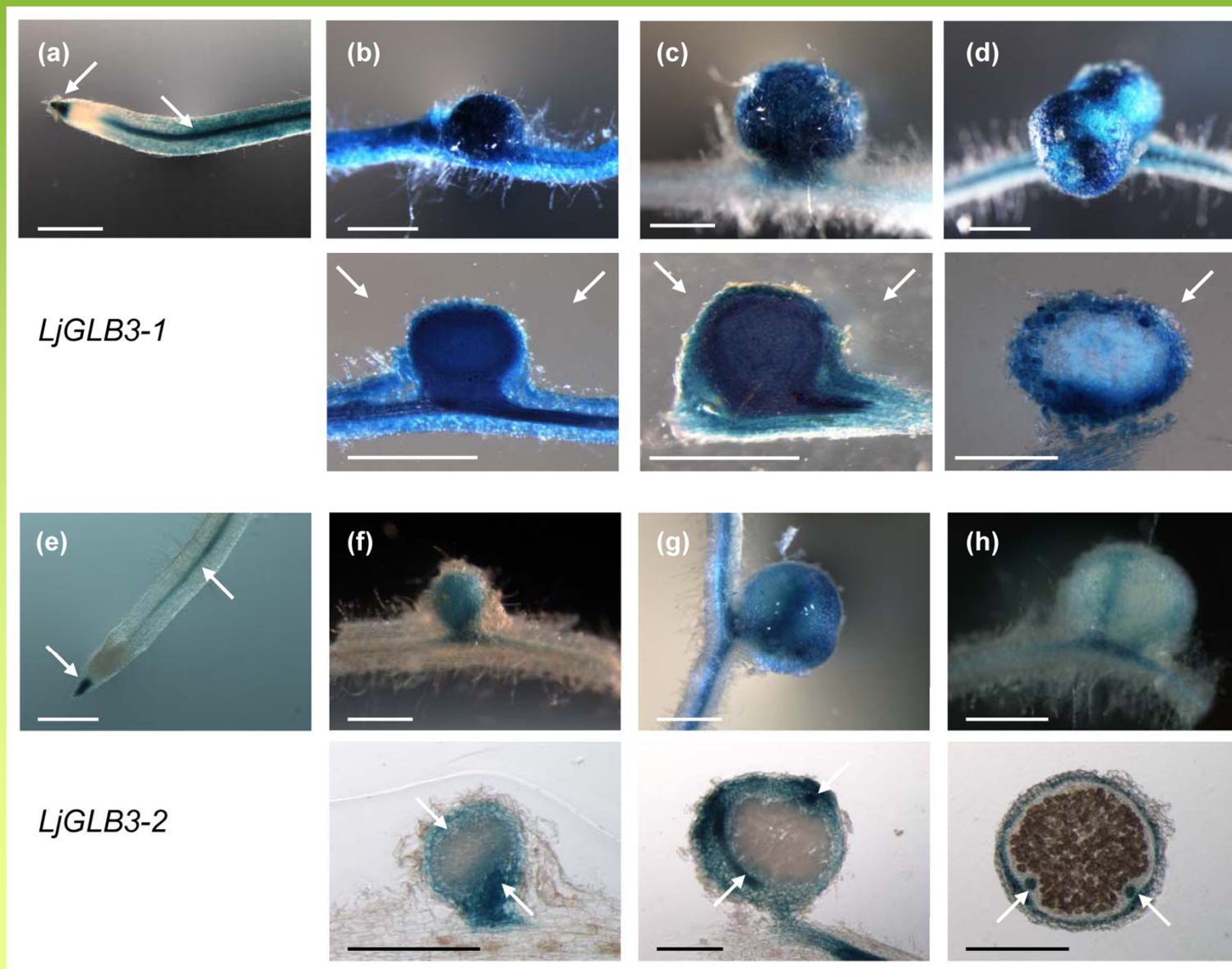
## Nonsymbiotic hemoglobin *LjGLB2*: promoter activity localization

- in roots, the *LjGLB2* promoter is active in the tips and vascular bundles
- in young nodules, the promoter is more active in the cortex, infected zone (especially in the periphery) and vascular bundles; however, as nodules age, staining can be seen only in the vascular bundles



## Truncated hemoglobins *LjGLB3-1* and *LjGLB3-2*: promoter activity localization

- in roots, the two promoters are active in the tips and vascular tissue
- in young nodules, the *LjGLB3-1* promoter is active in the cortex, infected zone, and vascular bundles; as nodules age, staining can be only seen in the mid/inner cortex and vascular bundles
- in nodules, *LjGLB3-2* promoter activity is seen in the mid-cortex and vascular bundles



## Conclusions and prospects

- **Superoxide dismutases** and **hemoglobins** are highly expressed in **vascular bundle cells**, which may be explained by the high metabolic activity of these cells associated with metabolite transport
- **GSH** and **hGSH** are synthesized in the **plastids** and **cytosol** of nodules, but  $\gamma$ EC needs to be exported from the plastids. The *GSHS* and *hGSHS* genes are differentially regulated in response to NO or hormones, suggesting **different functions for the two thiols**. Localization of promoter activities will be determined
- **GSNOR (LjADH4)** is expressed at significant levels in nodules and a novel **ADH (LjADH5)**, with perhaps intermediate properties between ADH and GSNOR, has been identified. Recombinant proteins are being prepared for biochemical characterization
- **Hemoglobins** GLB1-1, GLB2 and GLB3-1 are highly expressed in nodules and may be essential for symbiosis. GLB1-1 may be involved in **NO metabolism** (as occurs for the GLB1s of monocots), whereas GLB2 and GLB3-1 may play roles related to **nodule development**. Recombinant proteins and RNAi lines are being prepared for molecular and biochemical characterization

# Acknowledgments



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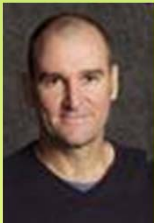
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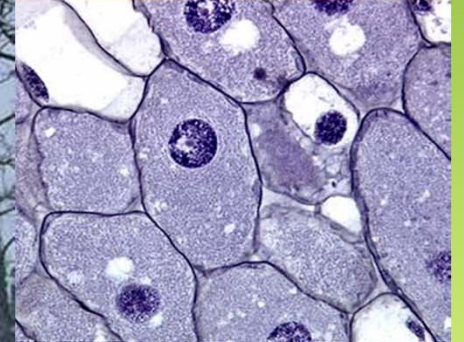
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Thank you for your attention!

