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Role of Heme Oxygenase in modulating expression of ROS-regulatory enzymes in Medicago truncatula

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ROLE OF HEME OXYGENASE IN MODULATING EXPRESSION OF ROS-REGULATORY ENZYMES IN *Medicago truncatula*

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ABSTRACT

Heme Oxygenase (HO) is an enzyme universally found in animals, plants and microbes. In plants, the role of heme oxygenase in the synthesis of the phytochrome chromophore is well recognized and has been extensively studied; however its role in regulating reactive oxygen species (ROS) in plants is just beginning to be explored, particularly in legumes. Legumes interact with *Rhizobium* bacteria to form symbiotic nitrogen fixing nodules. ROS plays an important role in the development of roots as well as symbiotic nodules. In the model legume *Medicago truncatula*, ROS in the root is regulated in part by the *LATD/NIP* gene. The *M. truncatula giraffe* mutant has a deletion that removes the entire HO coding sequence. We have found that the *M. truncatula GIRAFFE* HO regulates expression of some of the *LATD*/*NIP*-regulated ROS genes such as *RESPRATORY BURST OXIDASE HOMOLOG C (RBOHC)* and a cell wall peroxidase (*cwPRX2)* in seedlings. This means that the wild-type function of *GIRAFFE* is to up-regulate expression of *RBOHC* and *cwPRX2* in roots, in contrast to *LATD/NIP*, which down-regulates them. We also found that *LATD*/*NIP* and *GIRAFFE* do not regulate expression of each other in seedlings. Given that the highest expression of *GIRAFFE* HO is in a senescing nodule, we tested the expression of ROS-regulatory enzymes in senescing nodules. We found that *GIRAFFE* up-regulates expression of *RBOHC* during nitrate-induced nodule senescence. At present, with changing climatic conditions and exposure to various environmental stresses that can alter ROS homeostasis, characterizing the role of *GIRAFFE* in the antioxidant machinery of legumes can be useful in improving crop productivity and for enhancing soil fertility.

Dedication

This thesis is dedicated in sweet memory of my mother *Indrani Ghosh* and father

Tapan Ghosh

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Chapter 1: Introduction

Function of Heme Oxygenase in plants and animals

Heme Oxygenase (HO) catalyses the cleavage of heme to biliverdin with the release of equimolar amounts of iron and carbon monoxide (CO) (Tenhunen, Marver et al. 1968).

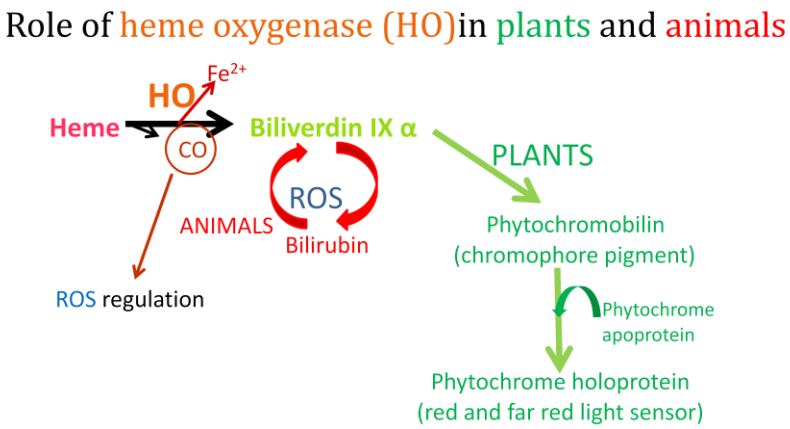


Figure 1: Heme Catabolism pathway in plants and animals. Heme is degraded to biliverdin by heme oxygenase. In plants biliverdin is a precursor in the synthesis of the phytochrome chromophore. In mammals, biliverdin is reduced to bilirubin, an antioxidant. HO, Heme Oxygenase; ROS, Reactive Oxygen Species; CO, Carbon Monoxide.

In mammals, biliverdin is reduced by biliverdin reductase (BVR) to bilirubin (BR) (Terry, Linley et al. 2002) which gets oxidized back into biliverdin (Baranano, Rao et al. 2002). An important role of the HO/BVR system has been established for neuronal disorders such as Alzheimer's disease (Barone, Di Domenico et al. 2013), cardiovascular diseases (Seki, Naruse et al. 1999) and diabetes (Abraham, Rezzani et al. 2004). In plants, HO is localized to the chloroplast, and the biliverdin it produces is a precursor for the synthesis of the phytochrome chromophore, phytochomobilin (Fig. 1) (Terry, Wahleithner et al. 1993). The phytochromobilin attaches to the phytochrome apoprotein, which is synthesised in the nucleus, to form the

phytochrome holoprotein, the red/far-red light sensor, which plays a significant role in photomorphogenesis (Terry 1997). In plants, recent literature suggests that CO regulates ROS levels in plant cells (Cui, Fu et al. 2011, Wu, Huang et al. 2011, Xie, Xu et al. 2011).

Today, the chloroplast is a permanent resident in a plant cell as a result of endosymbiosis of an ancestral cyanobacterium by a eukaryotic cell, about half a billion years ago (Lopez-Juez and Pyke 2005). The chloroplast still encodes for about a 100 proteins while the remaining 3000 proteins required for its biogenesis and function are encoded by the nucleus (Sato, Nakamura et al. 1999). This requires communicating signals from the chloroplast to the nucleus known as retrograde signaling (Nott, Jung et al. 2006). Heme oxygenase is one of the signaling molecules that influence transcription of the nuclear genome (Surpin, Larkin et al. 2002).

Heme Oxygenase gene in *Medicago truncatula*

Overview

Medicago truncatula has a single heme oxygenase gene, *GIRAFFE* (Medtr8g019320) (http://www.jcvi.org/medicago/ORF_infopage/23238/23238.m140766.html). The gene is 4 kb in size and is predicted to have four exons and three introns. The *giraffe* mutant was isolated in a fast neutron bombardment screen for plants with defects in both nodulation and development. The *giraffe* mutation is caused by a deletion of approximately 6.5 kb and is recessive (Zhang 2014).

Mutant phenotype in *Medicago truncatula*

The *giraffe* mutants have a photomorphogenic phenotype, with an elongated hypocotyl and petioles and yellowish-green leaves. Further, *giraffe* mutants fail to stimulate nodulation under red light or inhibit nodulation under far-red light (Zhang 2014). Co-segregation of the photomorphogenic and nodulation phenotypes and the deletion of the HO gene indicate that the *GIRAFFE HO* gene regulates both these traits (Zhang 2014).

Heme Oxygenase mutants in other species

In several plant species, heme oxygenase mutants have been identified that have a defect in the breakdown of heme to biliverdin and a characteristic photomorphogenic phenotype. In *Arabidopsis* photomorphogenic mutants with long hypocotyls that lack functional phytochrome chromophore have been characterised (Koornneef, Rolff et al. 1980, Chory, Peto et al. 1989). These mutants are deficient in a plastid-localised heme oxygenase that is encoded by the *HY1* gene (Davis, Kurepa et al. 1999, Muramoto, Kohchi et al. 1999). In pea the *phytochrome chromophore defective* mutant (*pcd1*), with etiolated seedlings and elongated hypocotyls, which has a defect in degrading heme to biliverdin (Weller, Terry et al. 1996), has a mutation in the pea heme oxygenase gene (*PsHO1*) (Linley, Landsberger et al. 2006). The phytochrome chromophore synthesis defective mutant in tomato, *yellow green-2 (yg-2)* is also defective in heme degradation to biliverdin (Terry and Kendrick 1996).

How is ROS produced and scavenged?

A shift in the redox status of the earth's atmosphere to an oxygen-rich environment (Bekker, Holland et al. 2004), required the evolution of protective antioxidant defence mechanisms against damage from reactive oxygen species for survival (Scandalios 2005). The levels of reactive oxygen species (ROS) are regulated by a network of ROS-producing and ROS-scavenging enzymes (Fig. 2).

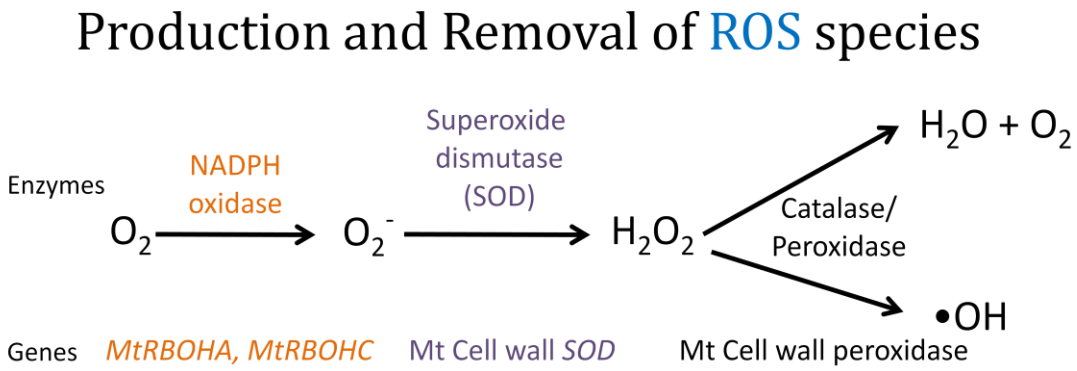


Figure 2: Cellular regulation of ROS intermediates via ROS-generating and scavenging enzymes.

Figure 2: Cellular regulation of ROS intermediates via ROS-generating and scavenging enzymes. Labels over the arrows indicate enzymes. Labels under the arrows are names of *Medicago truncatula* genes encoding those enzymes analyzed in this study.

Molecular oxygen is oxidized to superoxide anion (O_2^-), an ROS, by NADPH oxidases (Marino, Dunand et al. 2012). Subsequently, dismutation of the superoxide anion by superoxide dismutase (SOD) produces hydrogen peroxide (H_2O_2), another ROS (Fridovich 1995). Finally, catalases and peroxidases catalyse the decomposition of hydrogen peroxide to water and oxygen or to hydroxyl radical (Passardi, Penel et al. 2004, Zamocky, Gasselhuber et al. 2012).

Representatives of all these classes of enzymes are found in plants. The plant NADPH oxidases are known as *RESPIRATORY BURST OXIDASE HOMOLOGUES (RBOHS)* (Sagi and Fluhr 2001). These transmembrane proteins have an oxidase domain in their C-terminal region that oxidises molecular oxygen to superoxide anion. Similar to animal NADPH oxidases such as NOX5 and Duox, plant RBOHs have an N-terminal region containing EF hands, a motif that binds calcium (Grabarek 2006, Petry, Weitnauer et al. 2010, Suzuki, Miller et al. 2011). The EF hands and phosphorylation domain mark their importance in signal transduction pathways (Suzuki, Miller et al. 2011). Seven *RBOH* genes, *RBOHA-RBOHG*, have been identified in *Medicago truncatula* using protein sequence similarity with the Arabidopsis genome (Lohar, Haridas et al. 2007, Marino, Andrio et al. 2011).

The SODs are a large family of enzymes that can have mitochondrial, plastidial, cytosolic and apoplastic localizations (Asensio, Gil-Monreal et al. 2012). There can be three types of SODs depending on the co-factor used by the enzyme SOD; Iron SOD (FeSOD), Manganese SOD (MnSOD) and Copper-Zinc (Cu-ZnSOD) (Alscher, Erturk et al. 2002). The SODs act as the first line of defence in protecting cells from oxidative damage (Gill and Tuteja 2010).

The toxicity of hydrogen peroxide in plants is ameliorated chiefly by two enzymes, catalases and peroxidases (Welinder, Mauro et al. 1992, Willekens, Inze et al. 1995, Morgenstern, Klopman et al. 2008, Zamocky, Furtmuller et al. 2010), which have characteristic affinities for hydrogen peroxide. Catalases convert hydrogen peroxide to water and oxygen and function in defense responses and development especially germination and senescence (Willekens, Inze et al. 1995). The thiol based peroxidases, found in compartments such as the mitochondrion, chloroplast and the

nucleus, reduce hydrogen peroxide by the exchange of thiol and disulphide groups in their redox active cysteine residue (Dietz, Jacob et al. 2006, Puerto-Galan, Perez-Ruiz et al. 2013) and contribute to processes such as plastid metabolism and nodule development (Frendo, Matamoros et al. 2013, Michelet, Zaffagnini et al. 2013). The heme-containing peroxidases reduce hydrogen peroxide by the transfer of oxygen atom and electron between ferric and ferrous enzymes (Berglund, Carlsson et al. 2002). These are secreted into the cell wall or into the vacuole and have diverse functions ranging from environmental responses to developmental processes (Cosio and Dunand 2009, O'Brien, Daudi et al. 2012, Zipor and Oren-Shamir 2013).

Abiotic stress, HO and plants

The expression of heme oxygenase responds to various environmental stresses in different plant species. Heme Oxygenase has been found to be induced by abiotic stress such as salinity in *Arabidopsis* (Xie, Xu et al. 2011). In wheat seedlings, heme oxygenase expression is up regulated by chemicals such as hydrogen peroxide and by salinity (Xu, Jin et al. 2011). In soybean, HO is induced in leaves, roots and nodules by UV-B radiation and cadmium (Noriega, Balestrasse et al. 2004, Yannarelli, Noriega et al. 2006, Balestrasse, Yannarelli et al. 2008). The increased expression of HO in soybean leaves in response to salinity has been implicated in protecting leaves from salt stress (Zilli, Santa-Cruz et al. 2009). In alfalfa the amelioration of cadmium induced oxidative stress is effected by salicylic acid via heme oxygenase induction (Cui, Li et al. 2012).

Nodule senescence as part of development

Overview of nodulation

Nodules are highly specialized symbiotic organs formed under nitrogen-limiting conditions, on roots and occasionally on shoots of legumes, following an interaction between the eukaryotic legume species and its specific prokaryotic rhizobial partner (Sprent 2007, Markmann and Parniske 2009). This symbiosis contributes enormously to biological nitrogen fixation, wherein atmospheric nitrogen is reduced to ammonia by the prokaryotic nitrogenase enzyme complex (Bulen and LeComte 1966, Raymond, Siefert et al. 2004), of the rhizobial partner housed within the legume nodules (Virtanen and Miettinen 1963, Vance 2001, Dupont 2012). Legume nodules can be of two types; elongated, indeterminate nodules, such as those of *Medicago truncatula*, with an apical meristem, and globular, determinate nodules with no active meristem such as those of *Lotus japonicus* (Hirsch 1992). The initiation of nodulation begins with an exchange and recognition of chemical signals in the form Nod factors (NF) secreted by bacteria and flavonoid molecules produced by plants (Long 1996). Thereafter rhizobia become entrapped within a deformed and curled root hair, the plant cell wall degrades and cell membrane invaginates resulting in a growing apoplastic incipient tubule called infection thread (IT), populated by bacteria that grow and divide while in the IT (Gage and Margolin 2000, Gage 2004, Fournier, Timmers et al. 2008). Subsequent nodule organogenesis involves plant cortical cell division and differentiation, under tight genetic and hormonal regulation (Ding, Kalo et al. 2008, Den Herder and Parniske 2009, Popp and Ott 2011, Mortier, Holsters et

al. 2012). The developing IT ramifies, and spreads throughout the nodule primordium. The bacteria are eventually released from the tip of the IT into a young nodule cell as an infection droplet, with the surrounding plasma membrane becoming the peribacteroid membrane to form a compartment called the symbiosome. Within the symbiosome, the rhizobia terminally differentiate into bacteroids (Kereszt, Mergaert et al. 2011).

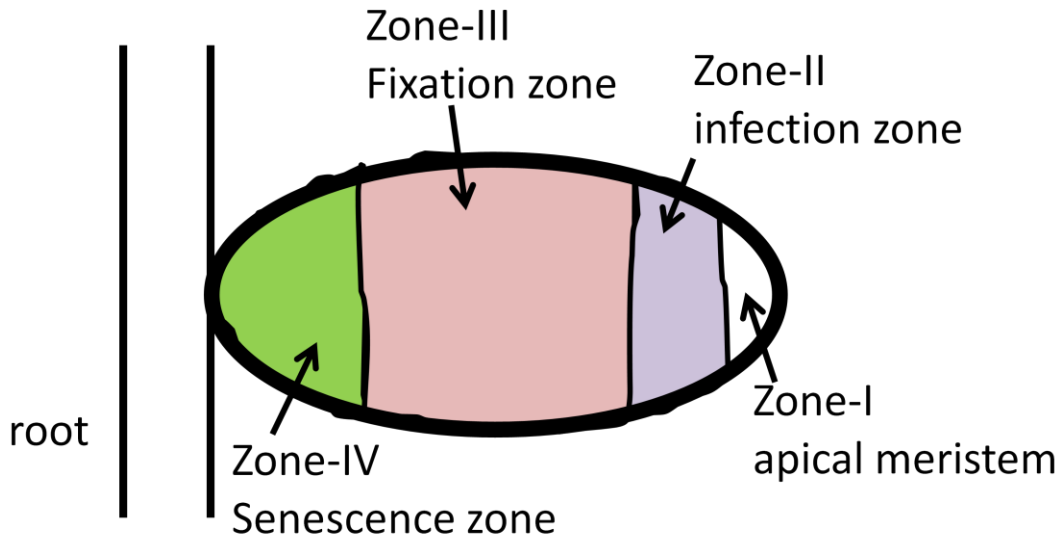


Figure 3: Schematic representation of developmental stages inside an indeterminate nodule

A symphony of intricate and coordinated cellular mechanisms in the nodule provides a microaerobic environmental niche to the nitrogenase enzyme complex of these bacteroids allowing them to fix atmospheric nitrogen to ammonia (Boyd and Peters 2013). The nitrogenase activity in the nodules begins to decline toward the end of flowering when pod filling begins (Klucas 1974). As part of the developmental biology of nodules, the process of nodule senescence sets in (Fig. 3), wherein a complex network of signaling pathways orchestrate the transition of the nodule from a carbon sink to a carbon source (Van de Velde, Guerra et al. 2006).

Nodule senescence

Physiologically, during nodule senescence, bacteroids get degraded, symbiosomes get resorbed, plant cells die and undifferentiated rhizobia in the infection thread get released into the soil (Paau, Bloch et al. 1980, Timmers, Soupene et al. 2000, Van de Velde, Guerra et al. 2006). The process of nodule senescence can be visually identified with a change in color from pink, nitrogen-fixing nodules to green, senescing nodules due to an irreversible oxidative degradation of pink-colored leghemoglobin into green-colored biliverdin (Virtanen and Miettinen 1963, Lehtovaara and Perttila 1978, Pfeiffer, Torres et al. 1983). Functionally, nodule senescence is characterized by a decrease in nitrogenase activity and an increase in proteolytic activity (Vance, Heichel et al. 1979, Malik, Pfeiffer et al. 1981). Proteolytic enzymes such as cysteine and papain proteases are implicated to play a role during nodule senescence (Florence., Mathis. Rene et al. 2003, Perez Guerra, Coussens et al. 2010, Pierre, Hopkins et al. 2014).

Environmental stresses such as salinity, drought, prolonged darkness and nitrate have a detrimental effect on nodule functioning and induce premature nodule senescence (Vauclare, Bligny et al. 2010, Mhadhbi, Dje´bali et al. 2011). It is long known that nitrate treatment is inversely related to the growth and the nitrogenase activity in nodules (Streeter 1981, Carroll and Gresshoff 1983, Streeter 1985, Streeter 1985). On nitrate exposure there is a decrease in the antioxidant milieu in the nodule that results in oxidative damage to proteins such as nitrogenase and leghemoglobin (Escuredo, Minchin et al. 1996).

ROS in the initiation and development of nodule formation

During the process of nodulation the levels of ROS in plant root cells in the nodulation zone is tightly regulated. In *M. sativa,* during infection there is accumulation of superoxide and hydrogen peroxide in the ITs and in the infected cells (Santos, Herouart et al. 2001). In *M. truncatula*, the perception of Nod factor and its function in regulating early nodulation gene expression requires signaling via ROS within 12 hours of inoculation (Ramu, Peng et al. 2002). Interestingly, root hair cells of *Phaseolus vulgaris* show a transient increase in ROS levels when treated with NFs (Cardenas, Martinez et al. 2008), and ROS production is required for the *M. truncatula* root hair cell curling, during initiation of the legume-rhizobia symbiosis (Peleg-Grossman, Volpin et al. 2007).

When *M. truncatula* roots are treated with NFs from its bacterial symbiont, *Sinorhizobium meliloti*, within 1 hour, the levels of *RBOH2* (*MtRBOHB*) and *RBOH3* (*MtRBOHE*) transcripts decrease transiently with a concomitant decrease in ROS, indicating a correlation between ROS levels and *RBOH* levels during infection (Shaw and Long 2003, Lohar, Haridas et al. 2007). In *Phaseolus vulgaris*, *PvRBOHB* expression correlates with ROS levels in roots that affects the initial stages of nodulation including structural deformities in ITs and anomalies in symbiosome structure, resulting in low nodule numbers and a drastic decrease in nitrogen fixation (Montiel, Nava et al. 2012, Arthikala, Sanchez-Lopez et al. 2014). The efficacy of nitrogen fixation in *M. truncatula* nodules correlates with the up-regulation of

MtRBOHA and is reduced in roots in which *MtRBOHA* expression is reduced by RNAi (Marino, Andrio et al. 2011).

The *LATERAL ROOT ORGAN DEFECTIVE/NUMEROUS INFECTIONS and POLYPHENOLICS* (*LATD/NIP)* gene in *M. truncatula* regulates superoxide levels in *M. truncatula* roots by regulating levels of *MtRBOHA, MtRBOHC and MtRBOHD* genes (Chang Zhang and Jeanne Harris, unpublished data). The *LATD/NIP* gene encodes a transmembrane nitrate transporter (Bagchi, Salehin et al. 2012). The *latd/nip* mutants have defects in root growth and in the development and functioning of nodules (Veereshlingam, Haynes et al. 2004, Bright, Liang et al. 2005).

ROS in nodule senescence

Overall, nodule senescence is characterized by significant shifts in the redox balance in the senescing nodule tissue (Dupont 2012). The auto-oxidation of leghemoglobin is correlated with levels of the ROS superoxide anion in nodules (Puppo, Rigaud et al. 1981) and lower leghemoglobin levels correlates with lower levels of hydrogen peroxide, another ROS (Gunther, Schlereth et al. 2007).

Beyond these observations, relatively little is known about the role of ROS during the process of nodule senescence, making this a fruitful area for investigation.

Interestingly, the highest expression of GIRAFFE HO is in a senescing nodule (*Medicago truncatula* Gene Expression Atlas; http://bioinfo.noble.org/gene-atlas/), and thus provides an entry point for studying ROS regulation in nodule senescence.

Summary

This thesis examines the role of *GIRAFFE* heme oxygenase of *Medicago truncatula* in modulating expression levels of ROS-regulatory enzymes in seedlings and in nitrate-induced senescing nodules. In this study, the expression of several genes that regulate levels of ROS and are modulated by the *LATD/NIP* gene has been assessed. Interestingly, the *LATD/NIP*-regulated *RBOHC* gene was found to also be regulated by the *GIRAFFE HO* in both seedlings and nitrate-induced senescing nodules and the *LATD/NIP*-regulated gene, *cwPRX2* was also found to be regulated by *GIRAFFE HO* in seedlings.

References

Abraham, N. G., R. Rezzani, L. Rodella, A. Kruger, D. Taller, G. Li Volti, A. I. Goodman and A. Kappas (2004). "Overexpression of human heme oxygenase-1 attenuates endothelial cell sloughing in experimental diabetes." Am J Physiol Heart Circ Physiol **287**(6): H2468-2477.

Alscher, R. G., N. Erturk and L. S. Heath (2002). "Role of superoxide dismutases (SODs) in controlling oxidative stress in plants." J Exp Bot **53**(372): 1331-1341.

Amicucci, E., K. Gaschler and J. M. Ward (1999). "NADPH oxidase genes from tomato (Lycopersicon esculentum) and curly-leaf pondweed (Potamogeton crispus)." Plant Biology **1**(5): 524-528.

Arthikala, M. K., J. Montiel, N. Nava, O. Santana, R. Sanchez-Lopez, L. Cardenas and C. Quinto (2013). "PvRbohB negatively regulates Rhizophagus irregularis colonization in Phaseolus vulgaris." Plant Cell Physiol **54**(8): 1391-1402.

Arthikala, M. K., R. Sanchez-Lopez, N. Nava, O. Santana, L. Cardenas and C. Quinto (2014). "RbohB, a Phaseolus vulgaris NADPH oxidase gene, enhances symbiosome number, bacteroid size, and nitrogen fixation in nodules and impairs mycorrhizal colonization." New Phytol **202**(3): 886-900.

Asensio, A. C., M. Gil-Monreal, L. Pires, Y. Gogorcena, P. M. Aparicio-Tejo and J. F. Moran (2012). "Two Fe-superoxide dismutase families respond differently to stress and senescence in legumes." J Plant Physiol **169**(13): 1253-1260.

Babior, B. M., J. D. Lambeth and W. Nauseef (2002). "The neutrophil NADPH oxidase." Archives of Biochemistry and Biophysics **397**(2): 342-344.

Bagchi, R., M. Salehin, O. S. Adeyemo, C. Salazar, V. Shulaev, D. J. Sherrier and R. Dickstein (2012). "Functional assessment of the Medicago truncatula NIP/LATD

protein demonstrates that it is a high-affinity nitrate transporter." Plant Physiol **160**(2): 906-916.
Balestrasse, K. B., G. G. Yannarelli, G. O. Noriega, A. Batlle and M. L. Tomaro (2008). "Heme oxygenase and catalase gene expression in nodules and roots of soybean plants subjected to cadmium stress." Biometals **21**(4): 433-441.
Bansal, S., G. Biswas and N. G. Avadhani (2014). "Mitochondria-targeted heme oxygenase-1 induces oxidative stress and mitochondrial dysfunction in macrophages, kidney fibroblasts and in chronic alcohol hepatotoxicity." Redox Biol **2**: 273-283.
Baranano, D. E., M. Rao, C. D. Ferris and S. H. Snyder (2002). "Biliverdin reductase: a major physiologic cytoprotectant." Proc Natl Acad Sci U S A **99**(25): 16093-16098.
Barone, E., F. Di Domenico, C. Mancuso and D. A. Butterfield (2013). "The Janus face of the heme oxygenase/biliverdin reductase system in Alzheimer disease: It's time for reconciliation." Neurobiol Dis.
Bekker, A., H. D. Holland, P. L. Wang, D. Rumble, H. J. Stein, J. L. Hannah, L. L. Coetzee and N. J. Beukes (2004). "Dating the rise of atmospheric oxygen." Nature **427**(6970): 117-120.
Berglund, G. I., G. H. Carlsson, A. T. Smith, H. Szoke, A. Henriksen and J. Hajdu (2002). "The catalytic pathway of horseradish peroxidase at high resolution." Nature **417**(6887): 463-468.
Boyd, E. S. and J. W. Peters (2013). "New insights into the evolutionary history of biological nitrogen fixation." Front Microbiol **4**: 201.
Bright, J., R. Desikan, J. T. Hancock, I. S. Weir and S. J. Neill (2006). "ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2 synthesis." Plant J **45**(1): 113-122.
Bright, L. J., Y. Liang, D. M. Mitchell and J. M. Harris (2005). "The LATD gene of Medicago truncatula is required for both nodule and root development." Mol Plant Microbe Interact **18**(6): 521-532.
Brown, S. B. and J. A. Holroyd (1984). "Biosynthesis of the chromophore of phycobiliproteins. A study of mesohaem and mesobiliverdin as possible intermediates and further evidence for an algal haem oxygenase." Biochem J **217**(1): 265-272.
Bulen, W. A. and J. R. LeComte (1966). "The nitrogenase system from Azotobacter: two-enzyme requirement for N2 reduction, ATP-dependent H2 evolution, and ATP hydrolysis." Proc Natl Acad Sci U S A **56**(3): 979-986.
Cabeza, R., B. Koester, R. Liese, A. Lingner, V. Baumgarten, J. Dirks, G. Salinas-Riester, C. Pommerenke, K. Dittert and J. Schulze (2014). "An RNA sequencing transcriptome analysis reveals novel insights into molecular aspects of the nitrate impact on the nodule activity of Medicago truncatula." Plant Physiol **164**(1): 400-411.
Cardenas, L., A. Martinez, F. Sanchez and C. Quinto (2008). "Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs)." Plant Journal **56**(5): 802-813.
Cardenas, L., A. Martinez, F. Sanchez and C. Quinto (2008). "Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs)." Plant J **56**(5): 802-813.
Carroll, B. J. and P. M. Gresshoff (1983). "Nitrate Inhibition of Nodulation and Nitrogen-Fixation in White Clover." Zeitschrift Fur Pflanzenphysiologie **110**(1): 77-88.
Chang, C., I. Damiani, A. Puppo and P. Frendo (2009). "Redox Changes during the Legume-Rhizobium Symbiosis." Molecular Plant **2**(3): 370-377.

Chory, J., C. A. Peto, M. Ashbaugh, R. Saganich, L. Pratt and F. Ausubel (1989). "Different Roles for Phytochrome in Etiolated and Green Plants Deduced from Characterization of Arabidopsis-Thaliana Mutants." Plant Cell **1**(9): 867-880.
Cosio, C. and C. Dunand (2009). "Specific functions of individual class III peroxidase genes." J Exp Bot **60**(2): 391-408.
Cui, W. T., G. Q. Fu, H. H. Wu and W. B. Shen (2011). "Cadmium-induced heme oxygenase-1 gene expression is associated with the depletion of glutathione in the roots of Medicago sativa." Biometals **24**(1): 93-103.
Cui, W. T., L. Li, Z. Z. Gao, H. H. Wu, Y. J. Xie and W. B. Shen (2012). "Haem oxygenase-1 is involved in salicylic acid-induced alleviation of oxidative stress due to cadmium stress in Medicago sativa." Journal of Experimental Botany **63**(15): 5521-5534.
Davis, S. J., S. H. Bhoo, A. M. Durski, J. M. Walker and R. D. Vierstra (2001). "The heme-oxygenase family required for phytochrome chromophore biosynthesis is necessary for proper photomorphogenesis in higher plants." Plant Physiol **126**(2): 656-669.
Davis, S. J., J. Kurepa and R. D. Vierstra (1999). "The Arabidopsis thaliana HY1 locus, required for phytochrome-chromophore biosynthesis, encodes a protein related to heme oxygenases." Proc Natl Acad Sci U S A **96**(11): 6541-6546.
Den Herder, G. and M. Parniske (2009). "The unbearable naivety of legumes in symbiosis." Curr Opin Plant Biol **12**(4): 491-499.
Dietz, K. J., S. Jacob, M. L. Oelze, M. Laxa, V. Tognetti, S. M. de Miranda, M. Baier and I. Finkemeier (2006). "The function of peroxiredoxins in plant organelle redox metabolism." J Exp Bot **57**(8): 1697-1709.
Ding, Y., P. Kalo, C. Yendrek, J. Sun, Y. Liang, J. F. Marsh, J. M. Harris and G. E. Oldroyd (2008). "Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodulation in Medicago truncatula." Plant Cell **20**(10): 2681-2695.
Dupont, L. A., G.;Pierre, O.; Msehli, S.; Hopkins, J.; Herouart, D.; Frendo, P. (2012). "The Legume Root Nodule: From Symbiotic Nitrogen Fixation to Senescence." Senescence; T. Nagata (ed.) Chapter 8: 137-168.
Ehrhardt, D. W., E. M. Atkinson and S. R. Long (1992). "Depolarization of alfalfa root hair membrane potential by Rhizobium meliloti Nod factors." Science **256**(5059): 998-1000.
Escuredo, P. R., F. R. Minchin, Y. Gogorcena, I. Iturbe-Ormaetxe, R. V. Klucas and M. Becana (1996). "Involvement of Activated Oxygen in Nitrate-Induced Senescence of Pea Root Nodules." Plant Physiol **110**(4): 1187-1195.
Florence., A., Mathis. Rene, G. Van de Skpe, D. Herouart and A. Puppo (2003). "Possible roles for cysteine protease and hydrogen peroxide in soybean nodule development and senescence." New Phyytologist **158**(1): 131-138.
Foreman, J., V. Demidchik, J. H. F. Bothwell, P. Mylona, H. Miedema, M. A. Torres, P. Linstead, S. Costa, C. Brownlee, J. D. G. Jones, J. M. Davies and L. Dolan (2003). "Reactive oxygen species produced by NADPH oxidase regulate plant cell growth." Nature **422**(6930): 442-446.
Fournier, J., A. C. Timmers, B. J. Sieberer, A. Jauneau, M. Chabaud and D. G. Barker (2008). "Mechanism of infection thread elongation in root hairs of Medicago truncatula and dynamic interplay with associated rhizobial colonization." Plant Physiol **148**(4): 1985-1995.

Frankenberg-Dinkel, N. (2004). "Bacterial heme oxygenases." Antioxid Redox Signal **6**(5): 825-834.
Frendo, P., M. A. Matamoros, G. Alloing and M. Becana (2013). "Thiol-based redox signaling in the nitrogen-fixing symbiosis." Front Plant Sci **4**: 376.
Fridovich, I. (1995). "Superoxide radical and superoxide dismutases." Annu Rev Biochem **64**: 97-112.
Gage, D. J. (2004). "Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes." Microbiol Mol Biol Rev **68**(2): 280-300.
Gage, D. J. and W. Margolin (2000). "Hanging by a thread: invasion of legume plants by rhizobia." Curr Opin Microbiol **3**(6): 613-617.
Gill, S. S. and N. Tuteja (2010). "Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants." Plant Physiol Biochem **48**(12): 909-930.
Golldack, D., C. Li, H. Mohan and N. Probst (2014). "Tolerance to drought and salt stress in plants: Unraveling the signaling networks." Front Plant Sci **5**: 151.
Grabarek, Z. (2006). "Structural basis for diversity of the EF-hand calcium-binding proteins." J Mol Biol **359**(3): 509-525.
Groom, Q. J., M. A. Torres, A. P. FordhamSkelton, K. E. HammondKosack, N. J. Robinson and J. D. G. Jones (1996). "rbohA a rice homologue of the mammalian gp91phox respiratory burst oxidase gene." Plant Journal **10**(3): 515-522.
Gunther, C., A. Schlereth, M. Udvardi and T. Ott (2007). "Metabolism of reactive oxygen species is attenuated in leghemoglobin-deficient nodules of Lotus japonicus." Mol Plant Microbe Interact **20**(12): 1596-1603.
Han, Y., J. Zhang, X. Chen, Z. Gao, W. Xuan, S. Xu, X. Ding and W. Shen (2008). "Carbon monoxide alleviates cadmium-induced oxidative damage by modulating glutathione metabolism in the roots of Medicago sativa." New Phytol **177**(1): 155-166.
Hirsch, A. M. (1992). "Developmental biology of legume nodulation." New Phytologist **122**(2): 211-237.
Kakar, K., M. Wandrey, T. Czechowski, T. Gaertner, W. R. Scheible, M. Stitt, I. Torres-Jerez, Y. Xiao, J. C. Redman, H. C. Wu, F. Cheung, C. D. Town and M. K. Udvardi (2008). "A community resource for high-throughput quantitative RT-PCR analysis of transcription factor gene expression in Medicago truncatula." Plant Methods **4**: 18.
Keller, T., H. G. Damude, D. Werner, P. Doerner, R. A. Dixon and C. Lamb (1998). "A plant homolog of the neutrophil NADPH oxidase gp91(phox) subunit gene encodes a plasma membrane protein with Ca2+ binding motifs." Plant Cell **10**(2): 255-266.
Kereszt, A., P. Mergaert and E. Kondorosi (2011). "Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims?" Mol Plant Microbe Interact **24**(11): 1300-1309.
Kleine, T., U. G. Maier and D. Leister (2009). "DNA transfer from organelles to the nucleus: the idiosyncratic genetics of endosymbiosis." Annu Rev Plant Biol **60**: 115-138.
Klucas, R. V. (1974). "Studies on soybean nodule senescence." Plant Physiol **54**(4): 612-616.

Koornneef, M., E. Rolff and C. J. P. Spruit (1980). "Genetic-Control of Light-Inhibited Hypocotyl Elongation in Arabidopsis-Thaliana (L) Heynh." Zeitschrift Fur Pflanzenphysiologie **100**(2): 147-160.
Kwak, J. M., I. C. Mori, Z. M. Pei, N. Leonhardt, M. A. Torres, J. L. Dangl, R. E. Bloom, S. Bodde, J. D. Jones and J. I. Schroeder (2003). "NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis." EMBO J **22**(11): 2623-2633.
Lehtovaara, P. and U. Perttila (1978). "Bile-pigment formation from different leghaemoglobins. Methine-bridge specificity of coupled oxidation." Biochem J **176**(2): 359-364.
Lin, Q., S. Weis, G. Yang, Y. H. Weng, R. Helston, K. Rish, A. Smith, J. Bordner, T. Polte, F. Gaunitz and P. A. Dennery (2007). "Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress." J Biol Chem **282**(28): 20621-20633.
Linley, P. J., M. Landsberger, T. Kohchi, J. B. Cooper and M. J. Terry (2006). "The molecular basis of heme oxygenase deficiency in the pcd1 mutant of pea." FEBS J **273**(12): 2594-2606.
Lohar, D. P., S. Haridas, J. S. Gantt and K. A. VandenBosch (2007). "A transient decrease in reactive oxygen species in roots leads to root hair deformation in the legume-rhizobia symbiosis." New Phytologist **173**(1): 39-49.
Long, S. R. (1996). "Rhizobium symbiosis: nod factors in perspective." Plant Cell **8**(10): 1885-1898.
Lopez-Juez, E. and K. A. Pyke (2005). "Plastids unleashed: their development and their integration in plant development." Int J Dev Biol **49**(5-6): 557-577.
Malik, N. S., N. E. Pfeiffer, D. R. Williams and F. W. Wagner (1981). "Peptidohydrolases of Soybean Root Nodules : IDENTIFICATION, SEPARATION, AND PARTIAL CHARACTERIZATION OF ENZYMES FROM BACTEROID-FREE EXTRACTS." Plant Physiol **68**(2): 386-392.
Marino, D., E. Andrio, E. G. Danchin, E. Oger, S. Gucciardo, A. Lambert, A. Puppo and N. Pauly (2011). "A Medicago truncatula NADPH oxidase is involved in symbiotic nodule functioning." New Phytol **189**(2): 580-592.
Marino, D., C. Dunand, A. Puppo and N. Pauly (2012). "A burst of plant NADPH oxidases." Trends Plant Sci **17**(1): 9-15.
Marino, D., C. Dunand, A. Puppo and N. Pauly (2012). "A burst of plant NADPH oxidases." Trends in Plant Science **17**(1): 9-15.
Markmann, K. and M. Parniske (2009). "Evolution of root endosymbiosis with bacteria: How novel are nodules?" Trends Plant Sci **14**(2): 77-86.
Martin, W., B. Stoebe, V. Goremykin, S. Hapsmann, M. Hasegawa and K. V. Kowallik (1998). "Gene transfer to the nucleus and the evolution of chloroplasts." Nature **393**(6681): 162-165.
Maruta, T., M. Noshi, A. Tanouchi, M. Tamoi, Y. Yabuta, K. Yoshimura, T. Ishikawa and S. Shigeoka (2012). "H2O2-triggered retrograde signaling from chloroplasts to nucleus plays specific role in response to stress." J Biol Chem **287**(15): 11717-11729.
Mathieu, C., S. Moreau, P. Frendo, A. Puppo and M. J. Davies (1998). "Direct detection of radicals in intact soybean nodules: presence of nitric oxide-leghemoglobin complexes." Free Radic Biol Med **24**(7-8): 1242-1249.
Mhadhbi, H., N. Dje´bali, S. Chihaoui, M. Jebara and R. Mhamdi (2011). "Nodule Senescence in Medicago truncatula–Sinorhizobium Symbiosis Under Abiotic

Constraints: Biochemical and Structural Processes Involved in Maintaining Nitrogen-Fixing Capacity " J Plant Growth Regulation **30**: 480-489.
Michelet, L., M. Zaffagnini, S. Morisse, F. Sparla, M. E. Perez-Perez, F. Francia, A. Danon, C. H. Marchand, S. Fermani, P. Trost and S. D. Lemaire (2013). "Redox regulation of the Calvin-Benson cycle: something old, something new." Front Plant Sci **4**: 470.
Mittler, R., S. Vanderauwera, N. Suzuki, G. Miller, V. B. Tognetti, K. Vandepoele, M. Gollery, V. Shulaev and F. Van Breusegem (2011). "ROS signaling: the new wave?" Trends Plant Sci **16**(6): 300-309.
Mochizuki, N., R. Tanaka, A. Tanaka, T. Masuda and A. Nagatani (2008). "The steady-state level of Mg-protoporphyrin IX is not a determinant of plastid-to-nucleus signaling in Arabidopsis." Proc Natl Acad Sci U S A **105**(39): 15184-15189.
Monshausen, G. B., T. N. Bibikova, M. A. Messerli, C. Shi and S. Gilroy (2007). "Oscillations in extracellular pH and reactive oxygen species modulate tip growth of Arabidopsis root hairs." Proc Natl Acad Sci U S A **104**(52): 20996-21001.
Montiel, J., M. K. Arthikala and C. Quinto (2013). "Phaseolus vulgaris RbohB functions in lateral root development." Plant Signal Behav **8**(1): e22694.
Montiel, J., N. Nava, L. Cardenas, R. Sanchez-Lopez, M. K. Arthikala, O. Santana, F. Sanchez and C. Quinto (2012). "A Phaseolus vulgaris NADPH oxidase gene is required for root infection by Rhizobia." Plant Cell Physiol **53**(10): 1751-1767.
Moreau, S., M. Verdenaud, T. Ott, S. Letort, F. de Billy, A. Niebel, J. Gouzy, F. de Carvalho-Niebel and P. Gamas (2011). "Transcription reprogramming during root nodule development in Medicago truncatula." PLoS One **6**(1): e16463.
Morgenstern, I., S. Klopman and D. S. Hibbett (2008). "Molecular evolution and diversity of lignin degrading heme peroxidases in the Agaricomycetes." J Mol Evol **66**(3): 243-257.
Mortier, V., M. Holsters and S. Goormachtig (2012). "Never too many? How legumes control nodule numbers." Plant Cell Environ **35**(2): 245-258.
Moulin, M., A. C. McCormac, M. J. Terry and A. G. Smith (2008). "Tetrapyrrole profiling in Arabidopsis seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-protoporphyrin IX accumulation." Proc Natl Acad Sci U S A **105**(39): 15178-15183.
Muramoto, T., T. Kohchi, A. Yokota, I. Hwang and H. M. Goodman (1999). "The Arabidopsis photomorphogenic mutant hy1 is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase." Plant Cell **11**(3): 335-348.
Noriega, G. O., K. B. Balestrasse, A. Batlle and M. L. Tomaro (2004). "Heme oxygenase exerts a protective role against oxidative stress in soybean leaves." Biochem Biophys Res Commun **323**(3): 1003-1008.
Nott, A., H. S. Jung, S. Koussevitzky and J. Chory (2006). "Plastid-to-nucleus retrograde signaling." Annu Rev Plant Biol **57**: 739-759.
O'Brien, J. A., A. Daudi, V. S. Butt and G. P. Bolwell (2012). "Reactive oxygen species and their role in plant defence and cell wall metabolism." Planta **236**(3): 765-779.
Oldroyd, G. E. and J. A. Downie (2008). "Coordinating nodule morphogenesis with rhizobial infection in legumes." Annu Rev Plant Biol **59**: 519-546.
Paau, A. S., C. B. Bloch and W. J. Brill (1980). "Developmental fate of Rhizobium meliloti bacteroids in alfalfa nodules." J Bacteriol **143**(3): 1480-1490.

Passardi, F., C. Penel and C. Dunand (2004). "Performing the paradoxical: how plant peroxidases modify the cell wall." Trends Plant Sci **9**(11): 534-540.
Peleg-Grossman, S., H. Volpin and A. Levine (2007). "Root hair curling and Rhizobium infection in Medicago truncatula are mediated by phosphatidylinositide-regulated endocytosis and reactive oxygen species." J Exp Bot **58**(7): 1637-1649.
Perez Guerra, J. C., G. Coussens, A. De Keyser, R. De Rycke, S. De Bodt, W. Van De Velde, S. Goormachtig and M. Holsters (2010). "Comparison of developmental and stress-induced nodule senescence in Medicago truncatula." Plant Physiol **152**(3): 1574-1584.
Petry, A., M. Weitnauer and A. Gorlach (2010). "Receptor activation of NADPH oxidases." Antioxid Redox Signal **13**(4): 467-487.
Pfeiffer, N. E., C. M. Torres and F. W. Wagner (1983). "Proteolytic Activity in Soybean Root Nodules : Activity in Host Cell Cytosol and Bacteroids throughout Physiological Development and Senescence." Plant Physiol **71**(4): 797-802.
Pierre, O., J. Hopkins, M. Combier, F. Baldacci, G. Engler, R. Brouquisse, D. Herouart and E. Boncompagni (2014). "Involvement of papain and legumain proteinase in the senescence process of Medicago truncatula nodules." New Phytol **202**(3): 849-863.
Popp, C. and T. Ott (2011). "Regulation of signal transduction and bacterial infection during root nodule symbiosis." Curr Opin Plant Biol **14**(4): 458-467.
Puerto-Galan, L., J. M. Perez-Ruiz, J. Ferrandez, B. Cano, B. Naranjo, V. A. Najera, M. Gonzalez, A. M. Lindahl and F. J. Cejudo (2013). "Overoxidation of chloroplast 2-Cys peroxiredoxins: balancing toxic and signaling activities of hydrogen peroxide." Front Plant Sci **4**: 310.
Puppo, A., K. Groten, F. Bastian, R. Carzaniga, M. Soussi, M. M. Lucas, M. R. de Felipe, J. Harrison, H. Vanacker and C. H. Foyer (2005). "Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process." New Phytologist **165**(3): 683-701.
Puppo, A., J. Rigaud and Job. D (1981). "Role of superoxide anion in leghemoglobin autooxidation." Plant Science Letters **22**: 353-360.
Ramel, F., C. Sulmon, M. Bogard, I. Couee and G. Gouesbet (2009). "Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in Arabidopsis thaliana plantlets." BMC Plant Biol **9**: 28.
Ramu, S. K., H. M. Peng and D. R. Cook (2002). "Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene rip1 in Medicago truncatula." Mol Plant Microbe Interact **15**(6): 522-528.
Raymond, J., J. L. Siefert, C. R. Staples and R. E. Blankenship (2004). "The natural history of nitrogen fixation." Mol Biol Evol **21**(3): 541-554.
Sagi, M., O. Davydov, S. Orazova, Z. Yesbergenova, R. Ophir, J. W. Stratmann and R. Fluhr (2004). "Plant respiratory burst oxidase homologs impinge on wound responsiveness and development in Lycopersicon esculentum." Plant Cell **16**(3): 616-628.
Sagi, M. and R. Fluhr (2001). "Superoxide production by plant homologues of the gp91(phox) NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection." Plant Physiology **126**(3): 1281-1290.
Sagi, M. and R. Fluhr (2006). "Production of reactive oxygen species by plant NADPH oxidases." Plant Physiol **141**(2): 336-340.

Santos, R., D. Herouart, S. Sigaud, D. Touati and A. Puppo (2001). "Oxidative burst in alfalfa-Sinorhizobium meliloti symbiotic interaction." Mol Plant Microbe Interact **14**(1): 86-89.
Sato, S., Y. Nakamura, T. Kaneko, E. Asamizu and S. Tabata (1999). "Complete structure of the chloroplast genome of Arabidopsis thaliana." DNA Res **6**(5): 283-290.
Scandalios, J. G. (2005). "Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses." Braz J Med Biol Res **38**(7): 995-1014.
Schmitt, M. P. (1997). "Utilization of host iron sources by Corynebacterium diphtheriae: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin." J Bacteriol **179**(3): 838-845.
Seki, T., M. Naruse, K. Naruse, T. Yoshimoto, A. Tanabe, M. Seki, K. Tago, T. Imaki, R. Demura and H. Demura (1999). "Induction of heme oxygenase produces load-independent cardioprotective effects in hypertensive rats." Life Sci **65**(10): 1077-1086.
Shaw, S. L. and S. R. Long (2003). "Nod factor elicits two separable calcium responses in Medicago truncatula root hair cells." Plant Physiol **131**(3): 976-984.
Sprent, J. I. (2007). "Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation." New Phytol **174**(1): 11-25.
Stocker, R., Y. Yamamoto, A. F. McDonagh, A. N. Glazer and B. N. Ames (1987). "Bilirubin is an antioxidant of possible physiological importance." Science **235**(4792): 1043-1046.
Streeter, J. G. (1981). "Effect of nitrate in the rooting medium on carbohydrate composition of soybean nodules." Plant Physiol **68**(4): 840-844.
Streeter, J. G. (1985). "Nitrate inhibition of legume nodule growth and activity : I. Long term studies with a continuous supply of nitrate." Plant Physiol **77**(2): 321-324.
Streeter, J. G. (1985). "Nitrate Inhibition of Legume Nodule Growth and Activity : II. Short Term Studies with High Nitrate Supply." Plant Physiol **77**(2): 325-328.
Sun, J., V. Cardoza, D. M. Mitchell, L. Bright, G. Oldroyd and J. M. Harris (2006). "Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation." Plant J **46**(6): 961-970.
Surpin, M., R. M. Larkin and J. Chory (2002). "Signal transduction between the chloroplast and the nucleus." Plant Cell **14 Suppl**: S327-338.
Suzuki, N., G. Miller, J. Morales, V. Shulaev, M. A. Torres and R. Mittler (2011). "Respiratory burst oxidases: the engines of ROS signaling." Curr Opin Plant Biol **14**(6): 691-699.
Tenhunen, R., H. S. Marver and R. Schmid (1968). "The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase." Proc Natl Acad Sci U S A **61**(2): 748-755.
Terry, M. J. (1997). "Phytochrome chromophore-deficient mutants." Plant Cell and Environment **20**(6): 740-745.
Terry, M. J. and R. E. Kendrick (1996). "The aurea and yellow-green-2 mutants of tomato are deficient in phytochrome chromophore synthesis." J Biol Chem **271**(35): 21681-21686.
Terry, M. J., P. J. Linley and T. Kohchi (2002). "Making light of it: the role of plant haem oxygenases in phytochrome chromophore synthesis." Biochem Soc Trans **30**(4): 604-609.

Terry, M. J., J. A. Wahleithner and J. C. Lagarias (1993). "Biosynthesis of the plant photoreceptor phytochrome." Arch Biochem Biophys **306**(1): 1-15.
Timmers, A. C., E. Soupene, M. C. Auriac, F. de Billy, J. Vasse, P. Boistard and G. Truchet (2000). "Saprophytic intracellular rhizobia in alfalfa nodules." Mol Plant Microbe Interact **13**(11): 1204-1213.
Timmis, J. N., M. A. Ayliffe, C. Y. Huang and W. Martin (2004). "Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes." Nat Rev Genet **5**(2): 123-135.
Torres, M. A., H. Onouchi, S. Hamada, C. Machida, K. E. Hammond-Kosack and J. D. Jones (1998). "Six Arabidopsis thaliana homologues of the human respiratory burst oxidase (gp91phox)." Plant J **14**(3): 365-370.
Van de Velde, W., J. C. Guerra, A. De Keyser, R. De Rycke, S. Rombauts, N. Maunoury, P. Mergaert, E. Kondorosi, M. Holsters and S. Goormachtig (2006). "Aging in legume symbiosis. A molecular view on nodule senescence in Medicago truncatula." Plant Physiol **141**(2): 711-720.
Van de Velde, W., J. C. P. Guerra, A. De Keyser, R. De Rycke, S. Rombauts, N. Maunoury, P. Mergaert, E. Kondorosi, M. Holsters and S. Goormachtig (2006). "Aging in legume symbiosis. A molecular view on nodule senescence in Medicago truncatula." Plant Physiology **141**(2): 711-720.
Vance, C. P. (2001). "Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources." Plant Physiol **127**(2): 390-397.
Vance, C. P., G. H. Heichel, D. K. Barnes, J. W. Bryan and L. E. Johnson (1979). "Nitrogen Fixation, Nodule Development, and Vegetative Regrowth of Alfalfa (Medicago sativa L.) following Harvest." Plant Physiol **64**(1): 1-8.
Vauclare, P., R. Bligny, E. Gout, V. De Meuron and F. Widmer (2010). "Metabolic and structural rearrangement during dark-induced autophagy in soybean (Glycine max L.) nodules: an electron microscopy and 31P and 13C nuclear magnetic resonance study." Planta **231**(6): 1495-1504.
Veereshlingam, H., J. G. Haynes, R. V. Penmetsa, D. R. Cook, D. J. Sherrier and R. Dickstein (2004). "nip, a symbiotic Medicago truncatula mutant that forms root nodules with aberrant infection threads and plant defense-like response." Plant Physiol **136**(3): 3692-3702.
Virtanen, A. I. and J. K. Miettinen (1963). "Biological Nitrogen Fixation " Plant Physiology, F.C. Steward (ed) Academic Press, New York **3**: 539-568.
Welinder, K. G., J. M. Mauro and L. Norskov-Lauritsen (1992). "Structure of plant and fungal peroxidases." Biochem Soc Trans **20**(2): 337-340.
Weller, J. L., M. J. Terry, C. Rameau, J. B. Reid and R. E. Kendrick (1996). "The Phytochrome-Deficient pcd1 Mutant of Pea Is Unable to Convert Heme to Biliverdin IX[alpha]." Plant Cell **8**(1): 55-67.
Willekens, H., D. Inze, M. Vanmontagu and W. Vancamp (1995). "Catalases in Plants." Molecular Breeding **1**(3): 207-228.
Wong, H. L., R. Pinontoan, K. Hayashi, R. Tabata, T. Yaeno, K. Hasegawa, C. Kojima, H. Yoshioka, K. Iba, T. Kawasaki and K. Shimamoto (2007). "Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension." Plant Cell **19**(12): 4022-4034.
Wu, M. Z., J. J. Huang, S. Xu, T. F. Ling, Y. J. Xie and W. B. Shen (2011). "Haem oxygenase delays programmed cell death in wheat aleurone layers by modulation of hydrogen peroxide metabolism." Journal of Experimental Botany **62**(1): 235-248.

Xie, Y. J., S. Xu, B. Han, M. Z. Wu, X. X. Yuan, Y. Han, Q. Gu, D. K. Xu, Q. Yang and W. B. Shen (2011). "Evidence of Arabidopsis salt acclimation induced by up-regulation of HY1 and the regulatory role of RbohD-derived reactive oxygen species synthesis." Plant J **66**(2): 280-292.
Xu, D. K., Q. J. Jin, Y. J. Xie, Y. H. Liu, Y. T. Lin, W. B. Shen and Y. J. Zhou (2011). "Characterization of a wheat heme oxygenase-1 gene and its responses to different abiotic stresses." Int J Mol Sci **12**(11): 7692-7707.
Xuan, W., S. Xu, X. Yuan and W. Shen (2008). "Carbon monoxide: A novel and pivotal signal molecule in plants?" Plant Signal Behav **3**(6): 381-382.
Yamamizo, C., K. Kuchimura, A. Kobayashi, S. Katou, K. Kawakita, J. D. Jones, N. Doke and H. Yoshioka (2006). "Rewiring mitogen-activated protein kinase cascade by positive feedback confers potato blight resistance." Plant Physiol **140**(2): 681-692.
Yannarelli, G. G., G. O. Noriega, A. Batlle and M. L. Tomaro (2006). "Heme oxygenase up-regulation in ultraviolet-B irradiated soybean plants involves reactive oxygen species." Planta **224**(5): 1154-1162.
Yendrek, C. R., Y. C. Lee, V. Morris, Y. Liang, C. I. Pislariu, G. Burkart, M. H. Meckfessel, M. Salehin, H. Kessler, H. Wessler, M. Lloyd, H. Lutton, A. Teillet, D. J. Sherrier, E. P. Journet, J. M. Harris and R. Dickstein (2010). "A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in Medicago truncatula." Plant J **62**(1): 100-112.
Yoshioka, H., N. Numata, K. Nakajima, S. Katou, K. Kawakita, O. Rowland, J. D. Jones and N. Doke (2003). "Nicotiana benthamiana gp91phox homologs NbrbohA and NbrbohB participate in H2O2 accumulation and resistance to Phytophthora infestans." Plant Cell **15**(3): 706-718.
Yoshioka, H., K. Sugie, H. J. Park, H. Maeda, N. Tsuda, K. Kawakita and N. Doke (2001). "Induction of plant gp91 phox homolog by fungal cell wall, arachidonic acid, and salicylic acid in potato." Mol Plant Microbe Interact **14**(6): 725-736.
Zamocky, M., P. G. Furtmuller and C. Obinger (2008). "Evolution of catalases from bacteria to humans." Antioxid Redox Signal **10**(9): 1527-1548.
Zamocky, M., P. G. Furtmuller and C. Obinger (2010). "Evolution of structure and function of Class I peroxidases." Arch Biochem Biophys **500**(1): 45-57.
Zamocky, M., B. Gasselhuber, P. G. Furtmuller and C. Obinger (2012). "Molecular evolution of hydrogen peroxide degrading enzymes." Arch Biochem Biophys **525**(2): 131-144.
Zhang, C., A. Bousquet and J. M. Harris (2014). "Abscisic acid and LATD/NIP modulate root elongation via reactive oxygen species in Medicago truncatula." Plant Physiol: DOI:10.1104/pp.1114.248542.
Zhang, Y. (2014). Light regulation of legume nodulation through a plant hormone signaling network, University of Vermont.
Zilli, C. G., D. M. Santa-Cruz, G. G. Yannarelli, G. O. Noriega, M. L. Tomaro and K. B. Balestrasse (2009). "Heme Oxygenase Contributes to Alleviate Salinity Damage in Glycine max L. Leaves." Int J Cell Biol **2009**: 848516.
Zipor, G. and M. Oren-Shamir (2013). "Do vacuolar peroxidases act as plant caretakers?" Plant Sci **199-200**: 41-47.

Chapter 2: Results

Introduction

The ubiquitious heme oxygenase (HO) enzyme catalyzes the breakdown of heme to biliverdin in bacteria, animals and plants (Tenhunen, Marver et al. 1968, Brown and Holroyd 1984, Frankenberg-Dinkel 2004). The oxidation of heme by bacterial HO releases iron that facilitates iron acquisition from heme by bacteria (Schmitt 1997). In animals, following the degradation of heme to biliverdin by heme oxygenase, biliverdin is converted by biliverdin reductase (BVR) to bilirubin, a powerful physiological antioxidant involved in regulating levels of reactive oxygen species (ROS) (Stocker, Yamamoto et al. 1987). In plants too, HO catalyzes the first and rate-limiting step of heme catabolism to biliverdin. Unlike animals, plants do not have a BVR enzyme and thus no bilirubin is produced; instead biliverdin serves as the precursor for the biosynthesis of the phytochrome chromophore (Terry, Wahleithner et al. 1993).

Reactive Oxygen Species (ROS) play important roles as signaling molecules during stress and in various developmental processes (Puppo, Groten et al. 2005, Mittler, Vanderauwera et al. 2011). Environmental stresses such as drought and salinity alter the cellular redox balance that trigger ROS-regulated pathways, which in turn facilitate adaptation of plants to the abiotic stress (Golldack, Li et al. 2014). ROS molecules such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) play crucial roles in regulating developmental and growth processes throughout the plant (Foreman, Demidchik et al. 2003, Cardenas, Martinez et al. 2008). Superoxide anion produced in aerobic biological systems is efficiently reduced

to hydrogen peroxide by superoxide dismutase (Fridovich 1995).The toxicity and the signaling potential of hydrogen peroxide is regulated by robust enzymatic mechanisms that degrade hydrogen peroxide to either to hydroxyl radical or to water and oxygen by peroxidases and catalases, respectively (Passardi, Penel et al. 2004, Zamocky, Furtmuller et al. 2008, Puerto-Galan, Perez-Ruiz et al. 2013).

The *RESPIRATORY BURST HEME OXYGENASE (RBOH)* genes encode plasma-membrane localized NADPH oxidases, related to animal NADPH oxidases such as NOX5 and Duox (Grabarek 2006, Petry, Weitnauer et al. 2010, Suzuki, Miller et al. 2011) that catalyze the reduction of oxygen to extracellular superoxide (Babior, Lambeth et al. 2002, Sagi and Fluhr 2006, Marino, Dunand et al. 2012).The *RBOH* genes play important roles in ROS signaling and development processes, promoting the length and density of lateral roots in *Phaseolus vulgaris* (Montiel, Arthikala et al. 2013), mediating the wound healing response in *Lycopersicum esculentum* (Sagi, Davydov et al. 2004) and cell wall growth in *Arabidopsis* (Monshausen, Bibikova et al. 2007).

In plants, altered levels of ROS in response to abiotic stresses such as ultraviolet radiation, salinity, and heavy metals are correlated with the induction of expression and activity of heme oxygenase (Noriega, Balestrasse et al. 2004, Yannarelli, Noriega et al. 2006, Han, Zhang et al. 2008, Wu, Huang et al. 2011). One of the *Arabidopsis* heme oxygenase enzymes, *LONG HYPOCOTYL 1* (*HY1*), functions to regulate expression of *RBOHD* to mediate the plant's response to salt acclimation by regulating levels of ROS (Xie, Xu et al. 2011). The induction of HO expression and activity ameliorates the cadmium-induced oxidative stress in *M. sativa* roots by up-regulating NADPH levels (Cui, Li et al. 2012). Increased expression of HO in the

aleurone layer of the wheat seed correlates with decreased amounts of the ROS molecule, hydrogen peroxide, resulting in a delay in programmed cell death during germination (Wu, Huang et al. 2011).

In *Medicago truncatula* the *GIRAFFE (GIR)* gene encodes the only HO in its genome (Zhang 2014), unlike *Arabidopsis* where three heme oxygenase genes (HY1, *AtHO3*, *AtHO4*) have been identified (Davis, Bhoo et al. 2001). The deletion of the entire coding region of the *GIR* HO in *giraffe* mutants results in a classic photomorphogenic phenotype, presumably due to a lack of phytochrome synthesis, and plants are thus insensitive to Red/Far-red light for both seedling growth and regulation of nodulation (Zhang 2014). The photomorphogenic phenotype of *giraffe* is very similar to that of the *hy1* mutant in *Arabidopsis* as well as that of the *phytochrome chromophore deficient 1* (*pcd1*) mutant in pea (Weller, Terry et al. 1996, Davis, Kurepa et al. 1999, Muramoto, Kohchi et al. 1999).

The roots of legumes are unique in that they form symbiotic associations with specific *Rhizobium* bacterial partners that result in the development of root nodules, highly specialized organs that function as a factory for biological nitrogen fixation by intracellular rhizobium by reducing atmospheric nitrogen to ammonia (Virtanen and Miettinen 1963, Hirsch 1992). At the molecular level, the recognition between the legume host and its rhizobial partner is effected via a chemical dialog between Nod factors secreted by the rhizobia and flavonoid signals secreted by legume roots (Oldroyd and Downie 2008). The levels of ROS are tightly regulated by a network of players during initiation, development, functioning and senescence of nodules in a manner not completely understood (Puppo, Groten et al. 2005).

At least seven *RBOH* genes have been identified in *Medicago truncatula* (Lohar, Haridas et al. 2007, Marino, Andrio et al. 2011). All seven genes are expressed in either roots or nodules or both. Due to independent gene duplication in different plant families and independent discoveries in different labs, similar gene names in different species do not correspond to orthologous genes. In *M. truncatula*, Mt*RBOHA* is expressed in the nitrogen-fixing zone of mature nodules and is required for nitrogen-fixation activity and the requirement of Py*RBOHB* in rhizobial infection in *Phaseolus vulgaris* has been established (Marino, Andrio et al. 2011, Arthikala, Montiel et al. 2013). The expression of Mt*RBOH2* and Mt*RBOH3* (which correspond to Mt*RBOHB* and Mt*RBOHE* (Marino, Andrio et al. 2011)) and levels of ROS is down-regulated in the first hour after treatment with Nod factors (Lohar, Haridas et al. 2007). The *LATERAL ROOT ORGAN DEFECTIVE/NUMEROUS INFECTIONS and POLYPHENOLICS* (*LATD/NIP*) gene, encodes a nitrate transporter, which is required for root and nodule development (Veereshlingam, Haynes et al. 2004, Bright, Liang et al. 2005, Yendrek, Lee et al. 2010, Bagchi, Salehin et al. 2012) as well as regulating the expression of *MtRBOHA*, *MtRBOHC* and *MtRBOHD* (Zhang, Bousquet et al. 2014). *MtRBOHC* expression, which is very low in *M. truncatula* roots (Marino, Andrio et al. 2011), is inversely correlated with root length (Zhang, Bousquet et al. 2014). High levels of *MtRBOHC* expression in *latd/nip* mutants are associated with decreased root elongation. A reduced level of *MtRBOHC* expression, either due to RNAi knockdown or down-regulation stimulated by the hormone Abscisic acid, causes increased root elongation (Zhang, Bousquet et al. 2014). *LATD/NIP* also regulates the expression of other ROS-related enzymes, Cu/Zn superoxide dismutase (*Cu/ZnSOD*) and cell wall peroxidase (cw*PRX2*).

The *giraffe* mutant gives us an opportunity to determine whether the GIRAFFE HO in *M. truncatula* is a component in the ROS-regulatory network both in seedlings and during nodule senescence. Our findings indicate that *GIRAFFE HO* regulates expression of *RBOHC* and a cell wall peroxidase that are involved in scavenging and regulating ROS levels during development and senescence.

Results

GIRAFFE regulates expression of *RBOHC* in seedling

In *Arabidopsis,* acclimation to salt stress is mediated by ROS levels that require the combined functions of *HY1* HO and *AtRBOHD* (Xie, Xu et al. 2011). We wondered whether the *Medicago HO*, *GIRAFFE,* might function similarly to regulate *RBOH* expression in *M. truncatula* during root growth. To test this, we compared expression of all seven *MtRBOH* genes in wild-type and *giraffe* seedlings in both root and shoot using qRT-PCR. We found that *RBOHC* expression is strongly down-regulated in *giraffe* mutant roots and significantly up-regulated in shoots (Fig. 4). In contrast, expression of the other *RBOH* genes was unaffected by the *giraffe* mutation (Fig. 4). Our results indicate that *GIRAFFE* function is required for normal *RBOHC* expression in roots and shoots, but not that of other *RBOH* genes.

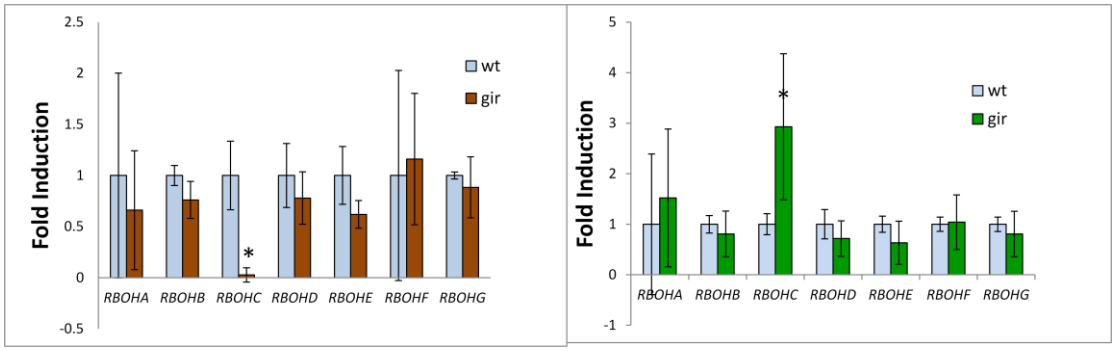


Figure 4: *GIRAFFE* regulates *RBOHC* expression in seedling roots and shoots

Roots and shoots were harvested at 6 days and *RBOH* gene expression analyzed by quantitative real-time RT-PCR. Values were normalized to *UBC* and *UBC9*. Error bars represent SEM between three biological replicates. n=10 plants per genotype for each replicate. Asterisks (*) represent a significant difference between *giraffe* and *wild-type* for $p < 0.05$ according to a student's t-test of the relative expression.

GIRAFFE regulates expression of *cwPRX2* but not cw*Cu/Zn SOD* in seedlings

The *LATD*/*NIP* gene up-regulates expression of *cwPRX2* and down-regulates that of *Cu/Zn SOD* (Zhang, Bousquet et al. 2014). We wondered whether *GIR* might also regulate the expression of these ROS-regulatory genes in seedlings, since both *LATD* and *GIRAFFE* regulate expression of the *MtRBOHC* gene. We compared the expression of these two genes in 6-day old roots and shoots in wild-type and *giraffe* mutants. Our results show that *cwPRX2* expression is down-regulated in *giraffe* roots and up-regulated in *giraffe* shoots, but expression of the *Cu/Zn SOD* is not significantly different from wild-type (Fig. 5). Thus, our results indicate that in seedling roots, *GIR* HO up-regulates the expression of *cwPRX2* but not that of *Cu/Zn SOD*.

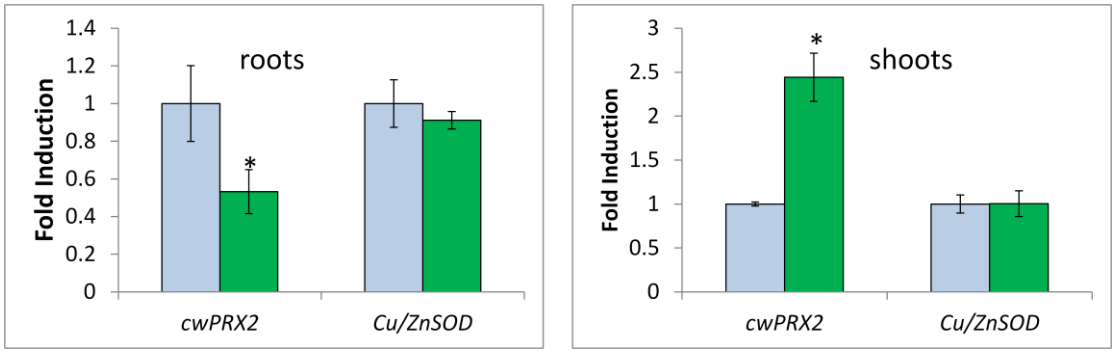


Figure 5: Regulation of ROS-regulatory genes by *GIRAFFE* in 6-day old roots and shoots

Plant tissue was harvested as described and gene expression analyzed by quantitative real-time RT-PCR and averaged from three biological replicates. Values are normalized to *UBC* and *UBC9*. Error bars indicate the SEM between three biological replicates. n = 10 for each genotype per replicate. Asterisks (*) represent a significant difference between *giraffe* and *wild-type* for $p < 0.05$ according to a student's t-test of the relative expression).

LATD/NIP and *GIRAFFE* do not regulate expression of each other

Our results show that in seedlings, *GIR* regulates the expression of *RBOHC* and *cwPRX2*. The regulation of these two genes by both *LATD/NIP* (Zhang, Bousquet et al. 2014) and *GIR* in roots could be due to their independent regulation by *GIR* and *LATD/NIP* (i.e. parallel pathways*)* or because *GIR* and *LATD/NIP* function in the same pathway. There are two possible linear pathways connecting *GIR* and *LATD*/*NIP*: a) *GIR* down-regulates *LATD/NIP*, which in turn down-regulates expression of the *MtRBOHC* and cwPRX2 genes in roots or b) *LATD/NIP* could down-regulate *GIR*, which is required to up-regulate the target genes. We tested the expression of *LATD/NIP* in *giraffe* mutants and that of *GIR* in *latd/nip* mutants and compared them with their expression in wild-type roots and shoots of 6-day old plants. We found no change either in the expression of the *LATD/NIP* gene in *giraffe*

mutants or in the expression of the *GIR* gene in *latd* mutants (Fig. 6), indicating that *GIR* and *LATD/NIP* do not regulate expression of each other in seedlings.

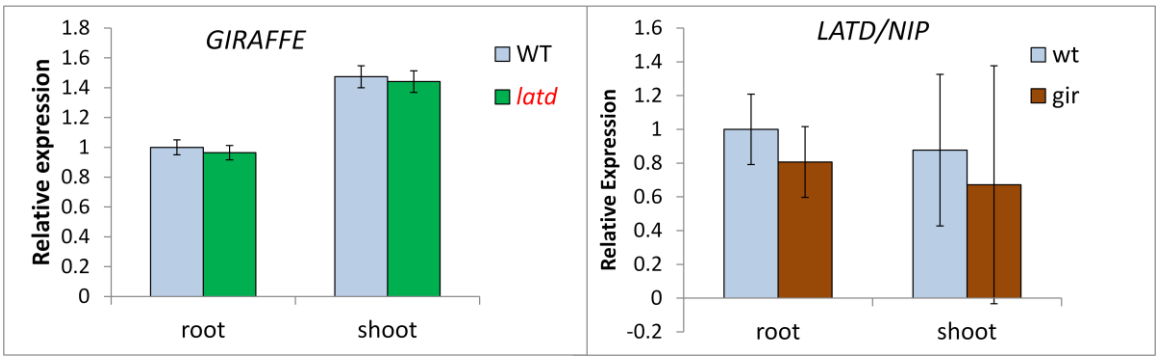


Figure 6: *GIRAFFE* and *LATD/NIP* do not regulate each other's expression

Seedling roots and shoots were pooled from 10 seedlings per genotype per replicate and gene expression analyzed by quantitative real-time RT-PCR. Values are normalized to *UBC* and *UBC9*. Error bars represent SEM between three biological replicates. Differences between genotypes are not significant, according to a t-test of the relative expression.

GIRAFFE regulates expression of *RBOHC* and *RBOHE* in senescing nodules

In functional symbiotic nodules of *M. truncatula*, the expression and upregulation of *MtRBOHA* is required for efficient nitrogen fixation (Marino, Andrio et al. 2011). Interestingly, the highest expression of *GIR*, which encodes a heme degrading enzyme, is in senescing nodules (Medicago truncatula Gene Expression Atlas (MtGEA); http://bioinfo.noble.org/gene-atlas/). We have found that *GIR* regulates expression of *RBOHC* in seedlings. Might *GIR* regulate *RBOH* expression in a senescing nodule, thereby playing a role in regulating ROS levels during the process of nodule senescence? We compared expression of all seven *RBOH* genes in both *giraffe* and wild-type mature, nitrogen-fixing nodules and in senescing nodules.

Nodule senescence was induced by treating once with 10 mM KNO_3 and senescing nodules were harvested from the top 10 cm of the root at 72 hours and gene expression was compared with that of a control group treated in parallel with 10 mM KCl. We found that *RBOHC* is induced during nitrate-induced senescence in wild-type but not *giraffe* nodules (Fig. 7). Instead, expression of *RBOHC* was significantly down-regulated in *giraffe* senescing nodules. In addition, we found that levels of *RBOHE* transcripts were also significantly lower in *giraffe* senescent nodules, although they were not significantly induced by nitrate treatment in wild-type nodules (Fig. 7). The expression of the other RBOH genes, *RBOHA, RBOHB, RBOHD, RBOHF* and *RBOHG,* were not altered significantly either by nitrate treatment in wild-type or in the mutants. In summary, our findings indicate that *GIR* functions to induce *RBOHC* and *RBOHE* expression in senescing nodules.

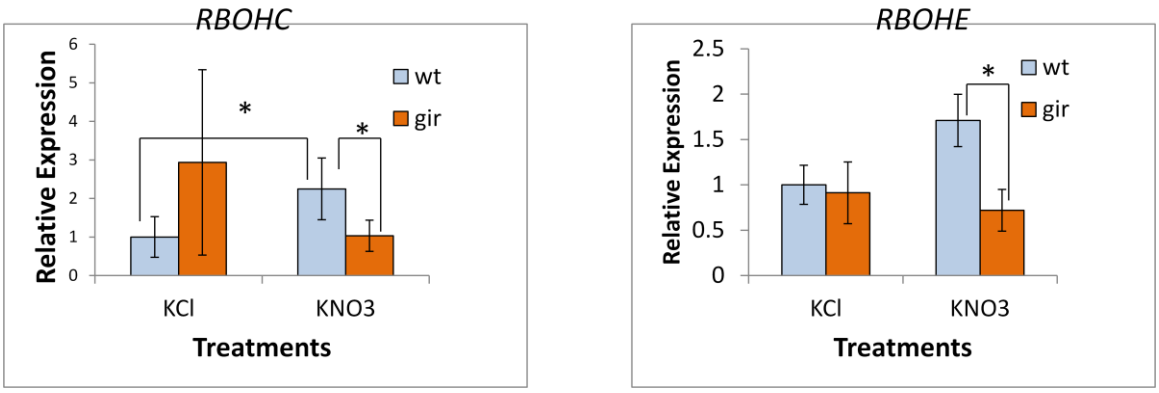
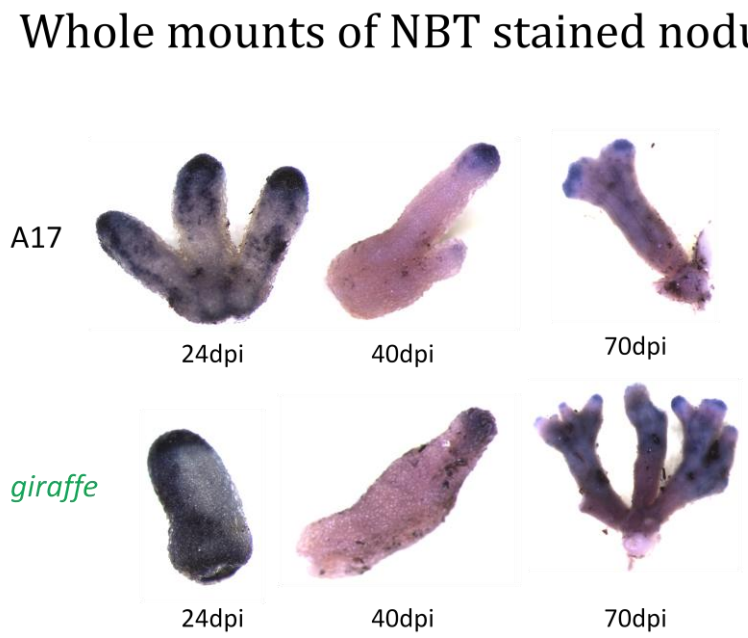


Figure 7: *GIRAFFE* regulates *RBOHC* and *RBOHE* expression in senescing nodules

Nodules were harvested 72 hours after treatment with either 10mM KCl (control) or 10mM KNO_3 (senescent) and gene expression analyzed by quantitative real-time RT-PCR. The average of three biological replicates is represented. Values are normalized to *UBC* and *UBC9.* Error bars represent the SEM between three biological replicates. Asterisks (*) represent a significant difference between genotypes or treatments, as indicated, for $p < 0.05$ according to the student's t-test of relative expression).

In the meristems of pea nodules, the levels of superoxide decrease as the nodules age (Puppo, Groten et al. 2005). Our results show that *GIR HO* regulates *RBOHC* and *RBOHE* expression in senescing nodules. We wondered if mutation in the *GIRAFFE HO* gene would show an altered superoxide distribution pattern in *Medicago* nodules at the time of senescence. We used Nitro Blue Tetrazolium (NBT) to stain for superoxide in whole and longitudinal sections of nodules at different stages of development in both wild-type and *giraffe* mutants (Fig. 8). We noted a gradual decrease in staining intensity, especially of the mersitem of mature nodules as they progress toward senescence. The amount of staining varied from plant to plant and we were unable to determine a clear difference in the pattern of staining between wild-type and *giraffe* nodules.



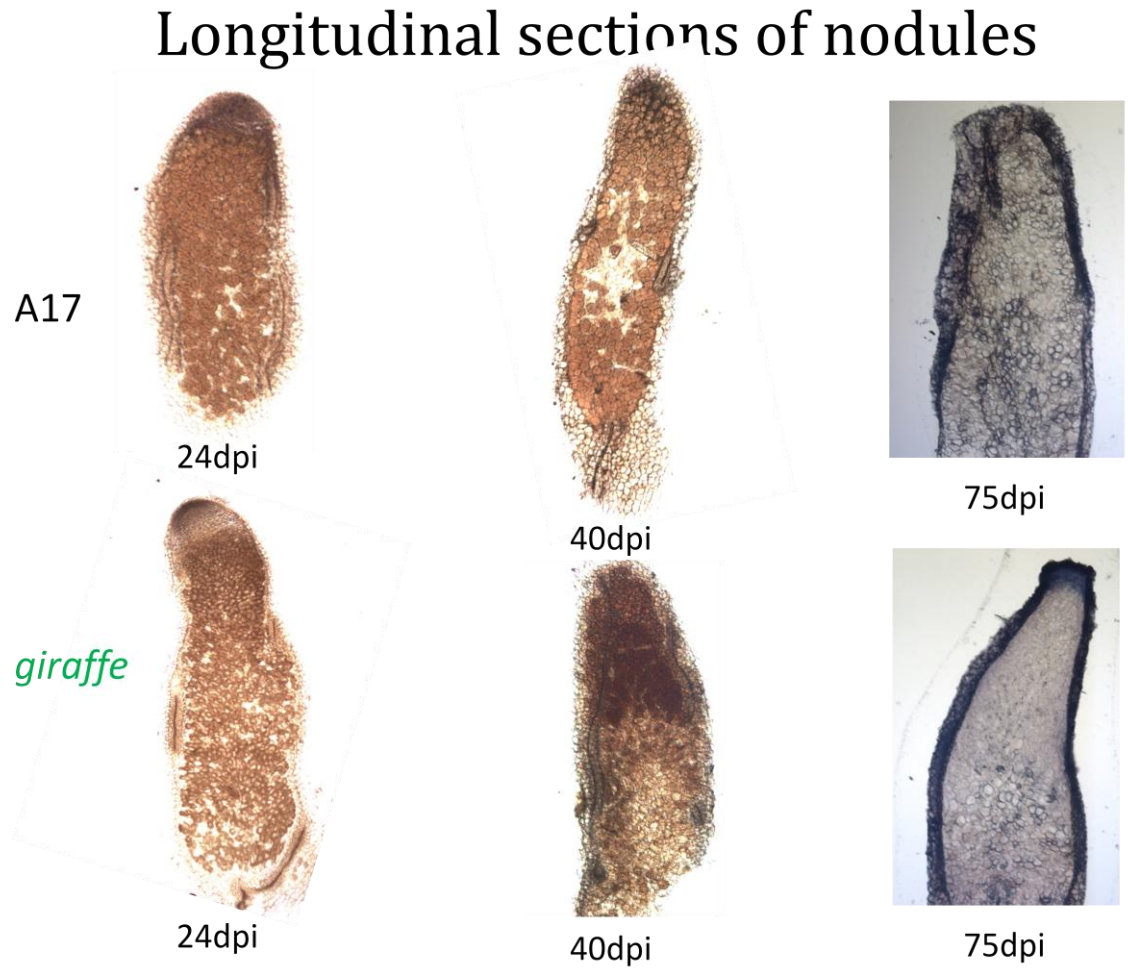


Figure 8: Whole mounts and longitudinal sections of nodules

Nodules were harvested from wild-type and giraffe mutants at 24dpi, 40dpi and 70 or 75dpi and they were stained with NBT for superoxide distribution in whole mounts and longitudinal section

GIRAFFE does not regulate leghemoglobin expression in the senescing nodule

The transcript levels of leghemoglobin (LB, a heme containing protein), is down-regulated in senescing nodules (Moreau, Verdenaud et al. 2011). We know that the expression of *GIR*, a heme degrading enzyme, is highest in a senescing nodule

(MtGEA; http://bioinfo.noble.org/gene-atlas/). Therefore we asked if *GIR* regulates expression of LB in a senescing nodule. Our results show that there is no significant change in *LB* expression between wild-type and *giraffe* mutants in mature or senescing nodules (Fig. 9). These observations suggest that *GIR* does not regulate *LB* expression in mature or senescing nodules.

GIRAFFE does not regulate *LATD/NIP* expression in senescing nodules

We have shown that *GIRAFFE HO* regulates expression of *RBOHC* and *RBOHE* in senescing nodules. Given that *GIRAFFE* expression is highest in a senescing nodule (MtGEA; http://bioinfo.noble.org/gene-atlas/) and the expression of *LATD/NIP*, which regulates expression of several *RBOH* genes in seedling roots (Zhang, Bousquet et al. 2014), is up-regulated in nodules four hours after nitrate treatment (Cabeza, Koester et al. 2014), we asked whether *GIRAFFE* regulates *RBOHC* expression in senescing nodules by controlling *LATD/NIP* expression. We examined the expression of *LATD/NIP* in senescing nodules of wild-type and *giraffe* mutants and found that the expression of *LATD/NIP* remains unaltered between wild-type and *giraffe* nodules whether or not they are induced to senesce (Fig. 9). Thus we conclude that *GIRAFFE* does not regulate RBOH gene expression by regulating *LATD/NIP* expression during nodule senescence.

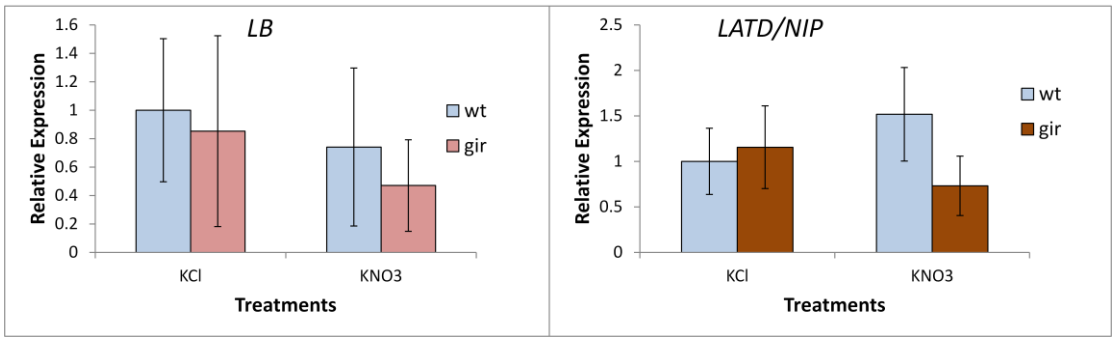


Figure 9: GIRAFFE does not regulate LB or LATD/NIP expression during nodule senescence

Nodules were harvested 72 hours after treatment with either 10 mM KCl (control) or 10 mM KNO3 (senescent) and gene expression was determined by quantitative real-time RT-PCR. The average gene expression values of three biological experiments is represented and normalized to *UBC* and *UBC9*. Error bars are SEM between three biological replicates.

Discussion

Our results show that the *GIR HO* of *M. truncatula* is involved in transcriptional regulation of *MtRBOHC* and *cwPRX2* in both roots and shoots. The other members of the superoxide-generating *RBOH* gene family and a *LATD/NIP*-regulated *Cu/ZnSOD* enzyme tested in this study were not found to be regulated by *GIR*. In nitrate-induced senescing nodules, *GIR* was found to again regulate the expression of *RBOHC* and in addition regulate expression of one other family member, *MtRBOHC*, but not that of any of the other *RBOH* genes or of LB.

Different RBOH genes have evolved unique functions in different developmental processes and stress responses.

The multigenic *RBOH* family is comprised of members with different spatio-temporal functions and regulations during plant development and stress responses, both biotic and abiotic. *RBOH* genes were first discovered in *Oryza sativa* (rice) (Groom, Torres

et al. 1996), where there are at least nine homologues (Wong, Pinontoan et al. 2007). Analysis of RBOH gene expression and function in several species demonstrate specific roles of specific RBOH genes in different species. In *Arabidopsis*, out of the ten paralogues (Keller, Damude et al. 1998, Torres, Onouchi et al. 1998, Kwak, Mori et al. 2003), *AtRBOHC* functions in the expansion of cell wall and root growth (Foreman, Demidchik et al. 2003) while *AtRBOHD* and *AtRBOHF* functions in stomatal closure induced by the plant hormone abscisic acid via nitric oxide signaling (Bright, Desikan et al. 2006). The *RBOH* homologs in *Lycopersicon esculentum* (tomato), *LeRBOH1* and *whitefly-induced* (*Wfi1),* are involved in regulating wound response genes and growth transcription factors (Sagi, Davydov et al. 2004) and *LeRBOH1* has the highest expressed in cold-stressed seedlings (Amicucci, Gaschler et al. 1999). At least four *RBOHs* have been identified in *Solanum tuberosum* (potato) and *StRBOHB* is induced by fungal cell wall elicitor while *StRBOHC* and *StRBOHD* are highly induced during late blight disease of potato (Yoshioka, Sugie et al. 2001, Yamamizo, Kuchimura et al. 2006). In *Nicotiana benthamiana* (tobacco), *NtRBOHA* and *NtRBOHB* are required in defense responses during fungal infection (Yoshioka, Numata et al. 2003).

A unique role for *MtRBOHC* in seedlings and nodules

Out of the seven RBOH homologues in *M. truncatula*, *RBOHC* is expressed at very low levels in roots and nodules compared to other RBOH family members (Marino, Andrio et al. 2011). We find that *RBOHC* is up-regulated by *GIR HO* in seedling roots and down-regulated in seedling shoots (Fig. 4). Interestingly, *RBOHC* expression is also regulated by *LATD/NIP* in the root but in the opposite way from *GIR*; the wild-type function of *LATD/NIP* is to reduce *RBOHC* expression (Zhang,

Bousquet et al. 2014). However, we found that *GIR* and *LATD/NIP* do not regulate the expression of each other in seedlings. The regulation of an otherwise less expressed *RBOHC* by *GIR HO* and *LATD/NIP* could indicate an important role of *RBOHC* in regulating ROS during cell wall synthesis (Monshausen, Bibikova et al. 2007). It is possible that *GIR* and *LATD* regulate cell wall synthesis by regulating ROS-regulatory enzymes such as *RBOHC* and cwPRX2 in roots. This is consistent with the observation that the cell length and primary root length is altered in both *gir* (Beck Powers and Jeanne Harris unpublished data) and *latd* (Bright, Liang et al. 2005, Zhang, Bousquet et al. 2014) mutants oppositely.

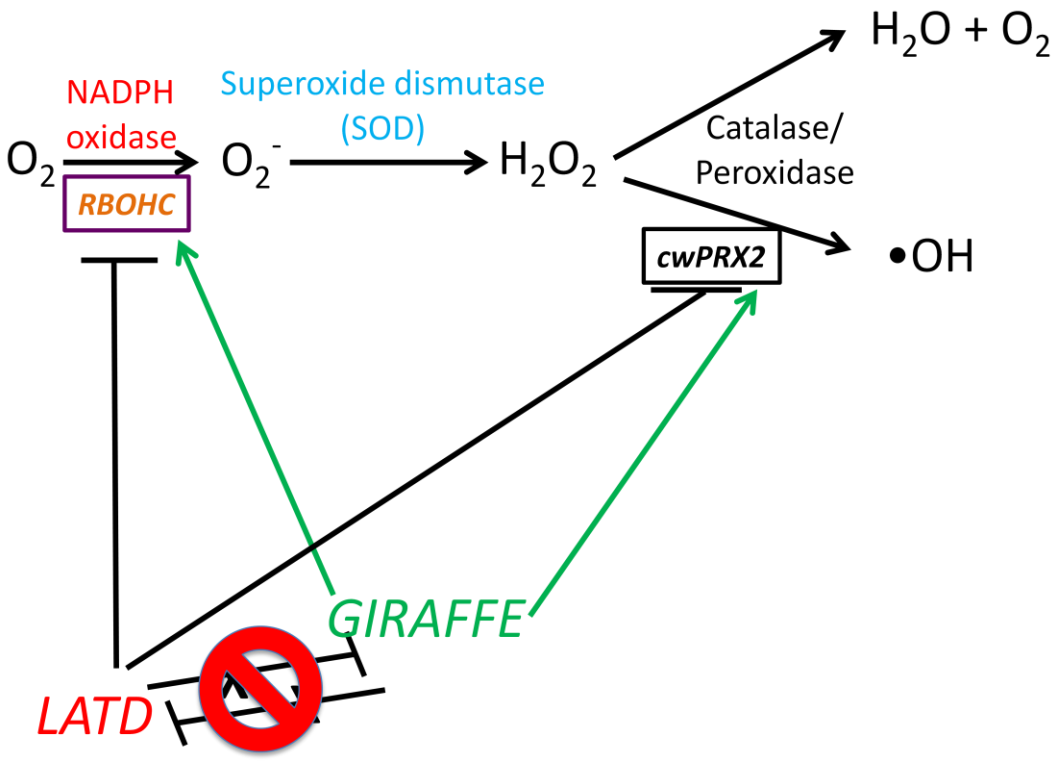


Figure 10: Summary of modulatory effects of *GIRAFFE* and *LATD/NIP*

GIRAFFE and *LATD/NIP* regulate expression of *RBOHC* and *cwPRX2* in opposite ways in *M. truncatula* seedlings

The development and functioning of legume nodules requires optimum levels of ROS and hence antioxidants and ROS levels are tightly regulated in nodules to maintain a

balance between N-fixation and damage by oxidative stress (Puppo, Groten et al. 2005, Chang, Damiani et al. 2009). When nodules senesce, leghemoglobin (pink) levels decrease (Pfeiffer, Torres et al. 1983) and the pink N-fixing nodule tissue turns into green senescent tissue as a result of an irreversible oxidation reaction (Virtanen and Miettinen 1963) due to breakdown of leghemoglobin (LB) into biliverdin (Lehtovaara and Perttila 1978). The auto-oxidation of LB is linked to the levels of superoxide anion in nodules (Puppo, Rigaud et al. 1981) and lower LB levels correlate with lower levels hydrogen peroxide (Gunther, Schlereth et al. 2007). Interestingly, the levels of nitric oxide-LB complex formed especially when nodules are treated with oxidant stress is comparable to those found in senescing nodules (Mathieu, Moreau et al. 1998). It would be interesting to investigate the oxidative modifications of leghemoglobin in the absence of *GIRAFFE* activity.

Role of GIRAFFE Heme Oxygenase in retrograde signaling

Chloroplasts have evolved from an ancestral free-living, photoautotrophic cyanobacterium that was endosymbiosed into a eukaryotic cell more than 450 million years ago (Lopez-Juez and Pyke 2005). The modern day eukaryotic plant cell has several functional nuclear genes of chloroplast origin (Martin, Stoebe et al. 1998, Timmis, Ayliffe et al. 2004, Kleine, Maier et al. 2009). Today, the highly reduced chloroplast genome barely encodes about a 100 proteins, the remaining 3000 proteins required for chloroplast biogenesis and function is encoded by the nuclear genome (Sato, Nakamura et al. 1999). This situation, requires chloroplast to nucleus signaling, known as retrograde signaling, to coordinate chloroplast development and function with nuclear gene expression (Surpin, Larkin et al. 2002, Mochizuki, Tanaka et al. 2008, Moulin, McCormac et al. 2008). Retrograde signaling can be triggered by ROS

molecules such as hydrogen peroxide (Maruta, Noshi et al. 2012). In animals under oxidative stress, the endoplasmic reticulum localized heme oxygenase translocates to the mitochondria or the nucleus to activate transcription factors (Lin, Weis et al. 2007, Bansal, Biswas et al. 2014). Our results show that in plants, the chloroplast-localized heme oxygenase regulates expression of cell wall localized ROS-regulatory enzymes. This implies that the chloroplast genome not only regulates expression of nuclear genes pertaining to its own biogenesis and function, but also of those involved in other cellular processes such as regulating ROS-regulating enzymes in the cell wall. It would be interesting to study the localization of heme oxygenase in plants under oxidative stress to study its signaling mechanism to the nucleus.

Materials and Methods

Plant Material and Growth Conditions

Seeds were scarified with 98% sulphuric acid, washed twice with distilled water and surface sterilized with 30% bleach (Clorox) followed by six washes in sterile water. Washed seeds were imbibed for 4-6 hours at room temperature, while shaking, then stored at 4° C overnight in sterile water and subsequently germinated overnight in an inverted petri plate sealed with parafilm in the dark at room temperature.

Harvesting seedlings: Seedlings were grown on filter paper (3M, cat no. 1530-0) laid over Nitrogen-free Buffered Nodulation Medium (BNM; (Ehrhardt, Atkinson et al. 1992)) solidified with 1.15 % agar in round 150mm X 15mm (Fisher Scientific Cat no. 08-757-14) petri plates with 20 plants per plate. Plates were sealed with surgical tape (Micropore 3M Cat. No. 1530-0) and the lower part of each plate was wrapped

with tin foil. Three biological replicates of plants were grown for six days in MTR30 Conviron growth chambers with 16 hr light period and 50% humidity at 20° C. Roots and shoots were harvested separately from a pooled sample of 10 plants in each replicate. The harvested tissue was frozen immediately in liquid nitrogen.

Harvesting nodules: Seeds were started and germinated for three biological replicates as described above, potted in $3\ ^{5}/_{8}$ -inch pots, in a mix of calcined clay (Profield Calcined Clay Soil Modifier): sand (all purpose)-1: 1 and grown in the greenhouse with temperature 64-66° C during day and 68-72° C at night. Supplemental lighting was provided to maintain 16 hour daylight period conditions at above 700µmol m^{-2} s^{-1}. Plants were fertilized 3 times a week with 20 ml of 1/2X BNM and watered with tap water as required. At 3 days, plants were inoculated with 5 ml of a 1:100 dilution of an overnight culture of *Sinorhizobium meliloti* 1021, grown in LB supplemented with 500µg/ml of Streptomycin and washed and diluted in 10 mM $MgSO_4$. After inoculation, plants were not watered until the following day. At 21 days post inoculation (dpi), plants were treated with 20 ml of either 10 mM KCl or 10 mM KNO_3. At 24dpi, nodules were harvested from the top 10 cm of the primary root, to yield approximately 0.5g fresh weight of nodule, and frozen immediately in liquid nitrogen.

Quantitative Real Time-PCR

Frozen tissue was ground over liquid nitrogen and total RNA extracted using the RNeasy Plant Mini kit (Qiagen Cat. No. 74904), DNase treated (Ambion Turbo DNA-free AM 1907) and cleaned using the Plant MinElute Clean-up kit (Qiagen Catalog no.74204). For first strand cDNA synthesis, 1µg RNA was reverse

transcribed using SuperScriptIII (Invitrogen), following the manufacturer's instructions. Quantitative Real Time-PCR was done on an ABI Step One qPCR machine with conditions for 20µl reactions set as 0.5µl cDNA, 0.1µM primers and 10µl PerfeCTA SYBR Green FastMix (Quanta Perfecta http://www.quantabio.com/). Gene expression was analysed using the ΔΔCT method, as in (Sun, Cardoza et al. 2006).

ROS staining, sectioning and microscopy

A modified protocol to stain nodules for superoxide with Nitroblue Tetrazolim (NBT) was developed from a protocol outlined by Ramu *et. al.* (Ramu, Peng et al. 2002). Nodules were incubated in a staining solution prepared with 10 mM sodium phosphate, 10 mM NaN_3 and 1mg/ml NBT (Sigma-Aldrich) for 15 minutes at 37°C. Two washes with 80% alcohol followed, to stop the staining reaction. Whole mounts were observed under Leica dissecting microscope. Nodules were longitudinally sectioned at 90µm thickness in a Lancer Vibratome Series 1000 and observed under Olympus microscope.

Statistical Analysis

Statistics were done using GraphPad Prism version 6.00 for windows. Expression data was analysed using Student's t-test on the logarithmic values of relative expression from three biological replicates.

Primers used in qRT-PCR

Gene	**Accession in Mt4.0**	**Forward Primer Sequence (5'-3')**	**Reverse Primer Sequence (5'-3')**	**Reference**
RbohA	Medtr1g083290	GCGCAACTCC TTTGATTAGC	GAAATAGGCT CGCTTGGTTG	(Marino, Andrio et al. 2011)
RbohB	Medtr3g098380	GCTCGCTCTGC TCTTATTGC	TGCGCTTGTA GACACTACGC	

RbohC	Medtr3g098350	GGGAGACCTGATGCTATTCAAG	TGTCTTCAACAATAATGTCCATCTG	
RbohD	Medtr3g098320	ACATGGCTCAGGAGCAAGAC	TGAAGAAGGCGTGGAAAGTC	
RbohE	Medtr8g095520	CCAAATTCTCACAGGCTTGC	CTCCATACGGACCATCAATC	
RbohF	Medtr7g060540	GAAGAGCCTTTGTCAAGAGTTTAG	GGATTGATGCTTTAGCTGTGTG	
RbohG	Medtr7g113130	GTGATGGATGAAATCGCAGA	TGGCATGATGAAGTGATTGA	
ApoCu/ZnSOD	Medtr6g029200	GGGCCTGCAAGAATGTTACC	CCTGAGGATGAGACTCGACA	(Zhang, Bousquet et al. 2014)
cwPRX2	Medtr2g084000	GGAAGAAGGGATAGTCTAGAGGCA	GGAGCTGGGACCTGGAAGAT	
LATD	Medtr1g009200	CCATAGCCTTTGGAGCAGAG	CTATCCCGATGCGGACTTTA	(Yendrek, Lee et al. 2010)
GIRAFFE	Medtr8g019320	TGGGAAGAAGATTGCTGGTC	TGTCCCTCACATTCTGCAAC	(Zhang 2014)
MtLB1	Medtr5g081000.1	TTGGGTGCTATCCACATTCA	TTCAGTTGCCAGTGCATCAT	
UBC	Medtr3g110110.1	CTGACAGCCCACTGAATTGTGA	TTTTGGCATTGCTGCAAGC	(Kakar, Wandrey et al. 2008)
UBC9	Medtr7g116940.1	GGTTGATTGCTCTTCTCTCCCC	AAGTGATTGCTCGTCCAACCC	

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References

Abraham, N. G., R. Rezzani, L. Rodella, A. Kruger, D. Taller, G. Li Volti, A. I. Goodman and A. Kappas (2004). "Overexpression of human heme oxygenase-1 attenuates endothelial cell sloughing in experimental diabetes." Am J Physiol Heart Circ Physiol **287**(6): H2468-2477.

Alscher, R. G., N. Erturk and L. S. Heath (2002). "Role of superoxide dismutases (SODs) in controlling oxidative stress in plants." J Exp Bot **53**(372): 1331-1341.

Amicucci, E., K. Gaschler and J. M. Ward (1999). "NADPH oxidase genes from tomato (Lycopersicon esculentum) and curly-leaf pondweed (Potamogeton crispus)." Plant Biology **1**(5): 524-528.

Arthikala, M. K., J. Montiel, N. Nava, O. Santana, R. Sanchez-Lopez, L. Cardenas and C. Quinto (2013). "PvRbohB negatively regulates Rhizophagus irregularis colonization in Phaseolus vulgaris." Plant Cell Physiol **54**(8): 1391-1402.

Arthikala, M. K., R. Sanchez-Lopez, N. Nava, O. Santana, L. Cardenas and C. Quinto (2014). "RbohB, a Phaseolus vulgaris NADPH oxidase gene, enhances symbiosome number, bacteroid size, and nitrogen fixation in nodules and impairs mycorrhizal colonization." New Phytol **202**(3): 886-900.

Asensio, A. C., M. Gil-Monreal, L. Pires, Y. Gogorcena, P. M. Aparicio-Tejo and J. F. Moran (2012). "Two Fe-superoxide dismutase families respond differently to stress and senescence in legumes." J Plant Physiol **169**(13): 1253-1260.

Babior, B. M., J. D. Lambeth and W. Nauseef (2002). "The neutrophil NADPH oxidase." Archives of Biochemistry and Biophysics **397**(2): 342-344.

Bagchi, R., M. Salehin, O. S. Adeyemo, C. Salazar, V. Shulaev, D. J. Sherrier and R. Dickstein (2012). "Functional assessment of the Medicago truncatula NIP/LATD protein demonstrates that it is a high-affinity nitrate transporter." Plant Physiol **160**(2): 906-916.

Balestrasse, K. B., G. G. Yannarelli, G. O. Noriega, A. Batlle and M. L. Tomaro (2008). "Heme oxygenase and catalase gene expression in nodules and roots of soybean plants subjected to cadmium stress." Biometals **21**(4): 433-441.

Bansal, S., G. Biswas and N. G. Avadhani (2014). "Mitochondria-targeted heme oxygenase-1 induces oxidative stress and mitochondrial dysfunction in macrophages, kidney fibroblasts and in chronic alcohol hepatotoxicity." Redox Biol **2**: 273-283.

Baranano, D. E., M. Rao, C. D. Ferris and S. H. Snyder (2002). "Biliverdin reductase: a major physiologic cytoprotectant." Proc Natl Acad Sci U S A **99**(25): 16093-16098.

Barone, E., F. Di Domenico, C. Mancuso and D. A. Butterfield (2013). "The Janus face of the heme oxygenase/biliverdin reductase system in Alzheimer disease: It's time for reconciliation." Neurobiol Dis.

Bekker, A., H. D. Holland, P. L. Wang, D. Rumble, H. J. Stein, J. L. Hannah, L. L. Coetzee and N. J. Beukes (2004). "Dating the rise of atmospheric oxygen." Nature **427**(6970): 117-120.

Berglund, G. I., G. H. Carlsson, A. T. Smith, H. Szoke, A. Henriksen and J. Hajdu (2002). "The catalytic pathway of horseradish peroxidase at high resolution." Nature **417**(6887): 463-468.
Boyd, E. S. and J. W. Peters (2013). "New insights into the evolutionary history of biological nitrogen fixation." Front Microbiol **4**: 201.
Bright, J., R. Desikan, J. T. Hancock, I. S. Weir and S. J. Neill (2006). "ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2 synthesis." Plant J **45**(1): 113-122.
Bright, L. J., Y. Liang, D. M. Mitchell and J. M. Harris (2005). "The LATD gene of Medicago truncatula is required for both nodule and root development." Mol Plant Microbe Interact **18**(6): 521-532.
Brown, S. B. and J. A. Holroyd (1984). "Biosynthesis of the chromophore of phycobiliproteins. A study of mesohaem and mesobiliverdin as possible intermediates and further evidence for an algal haem oxygenase." Biochem J **217**(1): 265-272.
Bulen, W. A. and J. R. LeComte (1966). "The nitrogenase system from Azotobacter: two-enzyme requirement for N2 reduction, ATP-dependent H2 evolution, and ATP hydrolysis." Proc Natl Acad Sci U S A **56**(3): 979-986.
Cabeza, R., B. Koester, R. Liese, A. Lingner, V. Baumgarten, J. Dirks, G. Salinas-Riester, C. Pommerenke, K. Dittert and J. Schulze (2014). "An RNA sequencing transcriptome analysis reveals novel insights into molecular aspects of the nitrate impact on the nodule activity of Medicago truncatula." Plant Physiol **164**(1): 400-411.
Cardenas, L., A. Martinez, F. Sanchez and C. Quinto (2008). "Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs)." Plant Journal **56**(5): 802-813.
Cardenas, L., A. Martinez, F. Sanchez and C. Quinto (2008). "Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs)." Plant J **56**(5): 802-813.
Carroll, B. J. and P. M. Gresshoff (1983). "Nitrate Inhibition of Nodulation and Nitrogen-Fixation in White Clover." Zeitschrift Fur Pflanzenphysiologie **110**(1): 77-88.
Chang, C., I. Damiani, A. Puppo and P. Frendo (2009). "Redox Changes during the Legume-Rhizobium Symbiosis." Molecular Plant **2**(3): 370-377.
Chory, J., C. A. Peto, M. Ashbaugh, R. Saganich, L. Pratt and F. Ausubel (1989). "Different Roles for Phytochrome in Etiolated and Green Plants Deduced from Characterization of Arabidopsis-Thaliana Mutants." Plant Cell **1**(9): 867-880.
Cosio, C. and C. Dunand (2009). "Specific functions of individual class III peroxidase genes." J Exp Bot **60**(2): 391-408.
Cui, W. T., G. Q. Fu, H. H. Wu and W. B. Shen (2011). "Cadmium-induced heme oxygenase-1 gene expression is associated with the depletion of glutathione in the roots of Medicago sativa." Biometals **24**(1): 93-103.
Cui, W. T., L. Li, Z. Z. Gao, H. H. Wu, Y. J. Xie and W. B. Shen (2012). "Haem oxygenase-1 is involved in salicylic acid-induced alleviation of oxidative stress due to cadmium stress in Medicago sativa." Journal of Experimental Botany **63**(15): 5521-5534.
Davis, S. J., S. H. Bhoo, A. M. Durski, J. M. Walker and R. D. Vierstra (2001). "The heme-oxygenase family required for phytochrome chromophore biosynthesis is necessary for proper photomorphogenesis in higher plants." Plant Physiol **126**(2): 656-669.

Davis, S. J., J. Kurepa and R. D. Vierstra (1999). "The Arabidopsis thaliana HY1 locus, required for phytochrome-chromophore biosynthesis, encodes a protein related to heme oxygenases." Proc Natl Acad Sci U S A **96**(11): 6541-6546.
Den Herder, G. and M. Parniske (2009). "The unbearable naivety of legumes in symbiosis." Curr Opin Plant Biol **12**(4): 491-499.
Dietz, K. J., S. Jacob, M. L. Oelze, M. Laxa, V. Tognetti, S. M. de Miranda, M. Baier and I. Finkemeier (2006). "The function of peroxiredoxins in plant organelle redox metabolism." J Exp Bot **57**(8): 1697-1709.
Ding, Y., P. Kalo, C. Yendrek, J. Sun, Y. Liang, J. F. Marsh, J. M. Harris and G. E. Oldroyd (2008). "Abscisic acid coordinates nod factor and cytokinin signaling during the regulation of nodulation in Medicago truncatula." Plant Cell **20**(10): 2681-2695.
Dupont, L. A., G.;Pierre, O.; Msehli, S.; Hopkins, J.; Herouart, D.; Frendo, P. (2012). "The Legume Root Nodule: From Symbiotic Nitrogen Fixation to Senescence." Senescence; T. Nagata (ed.) Chapter 8: 137-168.
Ehrhardt, D. W., E. M. Atkinson and S. R. Long (1992). "Depolarization of alfalfa root hair membrane potential by Rhizobium meliloti Nod factors." Science **256**(5059): 998-1000.
Escuredo, P. R., F. R. Minchin, Y. Gogorcena, I. Iturbe-Ormaetxe, R. V. Klucas and M. Becana (1996). "Involvement of Activated Oxygen in Nitrate-Induced Senescence of Pea Root Nodules." Plant Physiol **110**(4): 1187-1195.
Florence., A., Mathis. Rene, G. Van de Skpe, D. Herouart and A. Puppo (2003). "Possible roles for cysteine protease and hydrogen peroxide in soybean nodule development and senescence." New Phyytologist **158**(1): 131-138.
Foreman, J., V. Demidchik, J. H. F. Bothwell, P. Mylona, H. Miedema, M. A. Torres, P. Linstead, S. Costa, C. Brownlee, J. D. G. Jones, J. M. Davies and L. Dolan (2003). "Reactive oxygen species produced by NADPH oxidase regulate plant cell growth." Nature **422**(6930): 442-446.
Fournier, J., A. C. Timmers, B. J. Sieberer, A. Jauneau, M. Chabaud and D. G. Barker (2008). "Mechanism of infection thread elongation in root hairs of Medicago truncatula and dynamic interplay with associated rhizobial colonization." Plant Physiol **148**(4): 1985-1995.
Frankenberg-Dinkel, N. (2004). "Bacterial heme oxygenases." Antioxid Redox Signal **6**(5): 825-834.
Frendo, P., M. A. Matamoros, G. Alloing and M. Becana (2013). "Thiol-based redox signaling in the nitrogen-fixing symbiosis." Front Plant Sci **4**: 376.
Fridovich, I. (1995). "Superoxide radical and superoxide dismutases." Annu Rev Biochem **64**: 97-112.
Gage, D. J. (2004). "Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes." Microbiol Mol Biol Rev **68**(2): 280-300.
Gage, D. J. and W. Margolin (2000). "Hanging by a thread: invasion of legume plants by rhizobia." Curr Opin Microbiol **3**(6): 613-617.
Gill, S. S. and N. Tuteja (2010). "Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants." Plant Physiol Biochem **48**(12): 909-930.
Golldack, D., C. Li, H. Mohan and N. Probst (2014). "Tolerance to drought and salt stress in plants: Unraveling the signaling networks." Front Plant Sci **5**: 151.
Grabarek, Z. (2006). "Structural basis for diversity of the EF-hand calcium-binding proteins." J Mol Biol **359**(3): 509-525.

Groom, Q. J., M. A. Torres, A. P. FordhamSkelton, K. E. HammondKosack, N. J. Robinson and J. D. G. Jones (1996). "rbohA a rice homologue of the mammalian gp91phox respiratory burst oxidase gene." Plant Journal **10**(3): 515-522.
Gunther, C., A. Schlereth, M. Udvardi and T. Ott (2007). "Metabolism of reactive oxygen species is attenuated in leghemoglobin-deficient nodules of Lotus japonicus." Mol Plant Microbe Interact **20**(12): 1596-1603.
Han, Y., J. Zhang, X. Chen, Z. Gao, W. Xuan, S. Xu, X. Ding and W. Shen (2008). "Carbon monoxide alleviates cadmium-induced oxidative damage by modulating glutathione metabolism in the roots of Medicago sativa." New Phytol **177**(1): 155-166.
Hirsch, A. M. (1992). "Developmental biology of legume nodulation." New Phytologist **122**(2): 211-237.
Kakar, K., M. Wandrey, T. Czechowski, T. Gaertner, W. R. Scheible, M. Stitt, I. Torres-Jerez, Y. Xiao, J. C. Redman, H. C. Wu, F. Cheung, C. D. Town and M. K. Udvardi (2008). "A community resource for high-throughput quantitative RT-PCR analysis of transcription factor gene expression in Medicago truncatula." Plant Methods **4**: 18.
Keller, T., H. G. Damude, D. Werner, P. Doerner, R. A. Dixon and C. Lamb (1998). "A plant homolog of the neutrophil NADPH oxidase gp91(phox) subunit gene encodes a plasma membrane protein with Ca2+ binding motifs." Plant Cell **10**(2): 255-266.
Kereszt, A., P. Mergaert and E. Kondorosi (2011). "Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims?" Mol Plant Microbe Interact **24**(11): 1300-1309.
Kleine, T., U. G. Maier and D. Leister (2009). "DNA transfer from organelles to the nucleus: the idiosyncratic genetics of endosymbiosis." Annu Rev Plant Biol **60**: 115-138.
Klucas, R. V. (1974). "Studies on soybean nodule senescence." Plant Physiol **54**(4): 612-616.
Koornneef, M., E. Rolff and C. J. P. Spruit (1980). "Genetic-Control of Light-Inhibited Hypocotyl Elongation in Arabidopsis-Thaliana (L) Heynh." Zeitschrift Fur Pflanzenphysiologie **100**(2): 147-160.
Kwak, J. M., I. C. Mori, Z. M. Pei, N. Leonhardt, M. A. Torres, J. L. Dangl, R. E. Bloom, S. Bodde, J. D. Jones and J. I. Schroeder (2003). "NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis." EMBO J **22**(11): 2623-2633.
Lehtovaara, P. and U. Perttila (1978). "Bile-pigment formation from different leghaemoglobins. Methine-bridge specificity of coupled oxidation." Biochem J **176**(2): 359-364.
Lin, Q., S. Weis, G. Yang, Y. H. Weng, R. Helston, K. Rish, A. Smith, J. Bordner, T. Polte, F. Gaunitz and P. A. Dennery (2007). "Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress." J Biol Chem **282**(28): 20621-20633.
Linley, P. J., M. Landsberger, T. Kohchi, J. B. Cooper and M. J. Terry (2006). "The molecular basis of heme oxygenase deficiency in the pcd1 mutant of pea." FEBS J **273**(12): 2594-2606.

Lohar, D. P., S. Haridas, J. S. Gantt and K. A. VandenBosch (2007). "A transient decrease in reactive oxygen species in roots leads to root hair deformation in the legume-rhizobia symbiosis." New Phytologist **173**(1): 39-49.
Long, S. R. (1996). "Rhizobium symbiosis: nod factors in perspective." Plant Cell **8**(10): 1885-1898.
Lopez-Juez, E. and K. A. Pyke (2005). "Plastids unleashed: their development and their integration in plant development." Int J Dev Biol **49**(5-6): 557-577.
Malik, N. S., N. E. Pfeiffer, D. R. Williams and F. W. Wagner (1981). "Peptidohydrolases of Soybean Root Nodules : IDENTIFICATION, SEPARATION, AND PARTIAL CHARACTERIZATION OF ENZYMES FROM BACTEROID-FREE EXTRACTS." Plant Physiol **68**(2): 386-392.
Marino, D., E. Andrio, E. G. Danchin, E. Oger, S. Gucciardo, A. Lambert, A. Puppo and N. Pauly (2011). "A Medicago truncatula NADPH oxidase is involved in symbiotic nodule functioning." New Phytol **189**(2): 580-592.
Marino, D., C. Dunand, A. Puppo and N. Pauly (2012). "A burst of plant NADPH oxidases." Trends Plant Sci **17**(1): 9-15.
Marino, D., C. Dunand, A. Puppo and N. Pauly (2012). "A burst of plant NADPH oxidases." Trends in Plant Science **17**(1): 9-15.
Markmann, K. and M. Parniske (2009). "Evolution of root endosymbiosis with bacteria: How novel are nodules?" Trends Plant Sci **14**(2): 77-86.
Martin, W., B. Stoebe, V. Goremykin, S. Hapsmann, M. Hasegawa and K. V. Kowallik (1998). "Gene transfer to the nucleus and the evolution of chloroplasts." Nature **393**(6681): 162-165.
Maruta, T., M. Noshi, A. Tanouchi, M. Tamoi, Y. Yabuta, K. Yoshimura, T. Ishikawa and S. Shigeoka (2012). "H2O2-triggered retrograde signaling from chloroplasts to nucleus plays specific role in response to stress." J Biol Chem **287**(15): 11717-11729.
Mathieu, C., S. Moreau, P. Frendo, A. Puppo and M. J. Davies (1998). "Direct detection of radicals in intact soybean nodules: presence of nitric oxide-leghemoglobin complexes." Free Radic Biol Med **24**(7-8): 1242-1249.
Mhadhbi, H., N. Dje´bali, S. Chihaoui, M. Jebara and R. Mhamdi (2011). "Nodule Senescence in Medicago truncatula–Sinorhizobium Symbiosis Under Abiotic Constraints: Biochemical and Structural Processes Involved in Maintaining Nitrogen-Fixing Capacity " J Plant Growth Regulation **30**: 480-489.
Michelet, L., M. Zaffagnini, S. Morisse, F. Sparla, M. E. Perez-Perez, F. Francia, A. Danon, C. H. Marchand, S. Fermani, P. Trost and S. D. Lemaire (2013). "Redox regulation of the Calvin-Benson cycle: something old, something new." Front Plant Sci **4**: 470.
Mittler, R., S. Vanderauwera, N. Suzuki, G. Miller, V. B. Tognetti, K. Vandepoele, M. Gollery, V. Shulaev and F. Van Breusegem (2011). "ROS signaling: the new wave?" Trends Plant Sci **16**(6): 300-309.
Mochizuki, N., R. Tanaka, A. Tanaka, T. Masuda and A. Nagatani (2008). "The steady-state level of Mg-protoporphyrin IX is not a determinant of plastid-to-nucleus signaling in Arabidopsis." Proc Natl Acad Sci U S A **105**(39): 15184-15189.
Monshausen, G. B., T. N. Bibikova, M. A. Messerli, C. Shi and S. Gilroy (2007). "Oscillations in extracellular pH and reactive oxygen species modulate tip growth of Arabidopsis root hairs." Proc Natl Acad Sci U S A **104**(52): 20996-21001.
Montiel, J., M. K. Arthikala and C. Quinto (2013). "Phaseolus vulgaris RbohB functions in lateral root development." Plant Signal Behav **8**(1): e22694.

Montiel, J., N. Nava, L. Cardenas, R. Sanchez-Lopez, M. K. Arthikala, O. Santana, F. Sanchez and C. Quinto (2012). "A Phaseolus vulgaris NADPH oxidase gene is required for root infection by Rhizobia." Plant Cell Physiol **53**(10): 1751-1767.
Moreau, S., M. Verdenaud, T. Ott, S. Letort, F. de Billy, A. Niebel, J. Gouzy, F. de Carvalho-Niebel and P. Gamas (2011). "Transcription reprogramming during root nodule development in Medicago truncatula." PLoS One **6**(1): e16463.
Morgenstern, I., S. Klopman and D. S. Hibbett (2008). "Molecular evolution and diversity of lignin degrading heme peroxidases in the Agaricomycetes." J Mol Evol **66**(3): 243-257.
Mortier, V., M. Holsters and S. Goormachtig (2012). "Never too many? How legumes control nodule numbers." Plant Cell Environ **35**(2): 245-258.
Moulin, M., A. C. McCormac, M. J. Terry and A. G. Smith (2008). "Tetrapyrrole profiling in Arabidopsis seedlings reveals that retrograde plastid nuclear signaling is not due to Mg-protoporphyrin IX accumulation." Proc Natl Acad Sci U S A **105**(39): 15178-15183.
Muramoto, T., T. Kohchi, A. Yokota, I. Hwang and H. M. Goodman (1999). "The Arabidopsis photomorphogenic mutant hy1 is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase." Plant Cell **11**(3): 335-348.
Noriega, G. O., K. B. Balestrasse, A. Batlle and M. L. Tomaro (2004). "Heme oxygenase exerts a protective role against oxidative stress in soybean leaves." Biochem Biophys Res Commun **323**(3): 1003-1008.
Nott, A., H. S. Jung, S. Koussevitzky and J. Chory (2006). "Plastid-to-nucleus retrograde signaling." Annu Rev Plant Biol **57**: 739-759.
O'Brien, J. A., A. Daudi, V. S. Butt and G. P. Bolwell (2012). "Reactive oxygen species and their role in plant defence and cell wall metabolism." Planta **236**(3): 765-779.
Oldroyd, G. E. and J. A. Downie (2008). "Coordinating nodule morphogenesis with rhizobial infection in legumes." Annu Rev Plant Biol **59**: 519-546.
Paau, A. S., C. B. Bloch and W. J. Brill (1980). "Developmental fate of Rhizobium meliloti bacteroids in alfalfa nodules." J Bacteriol **143**(3): 1480-1490.
Passardi, F., C. Penel and C. Dunand (2004). "Performing the paradoxical: how plant peroxidases modify the cell wall." Trends Plant Sci **9**(11): 534-540.
Peleg-Grossman, S., H. Volpin and A. Levine (2007). "Root hair curling and Rhizobium infection in Medicago truncatula are mediated by phosphatidylinositide-regulated endocytosis and reactive oxygen species." J Exp Bot **58**(7): 1637-1649.
Perez Guerra, J. C., G. Coussens, A. De Keyser, R. De Rycke, S. De Bodt, W. Van De Velde, S. Goormachtig and M. Holsters (2010). "Comparison of developmental and stress-induced nodule senescence in Medicago truncatula." Plant Physiol **152**(3): 1574-1584.
Petry, A., M. Weitnauer and A. Gorlach (2010). "Receptor activation of NADPH oxidases." Antioxid Redox Signal **13**(4): 467-487.
Pfeiffer, N. E., C. M. Torres and F. W. Wagner (1983). "Proteolytic Activity in Soybean Root Nodules : Activity in Host Cell Cytosol and Bacteroids throughout Physiological Development and Senescence." Plant Physiol **71**(4): 797-802.
Pierre, O., J. Hopkins, M. Combier, F. Baldacci, G. Engler, R. Brouquisse, D. Herouart and E. Boncompagni (2014). "Involvement of papain and legumain

proteinase in the senescence process of Medicago truncatula nodules." New Phytol **202**(3): 849-863.
Popp, C. and T. Ott (2011). "Regulation of signal transduction and bacterial infection during root nodule symbiosis." Curr Opin Plant Biol **14**(4): 458-467.
Puerto-Galan, L., J. M. Perez-Ruiz, J. Ferrandez, B. Cano, B. Naranjo, V. A. Najera, M. Gonzalez, A. M. Lindahl and F. J. Cejudo (2013). "Overoxidation of chloroplast 2-Cys peroxiredoxins: balancing toxic and signaling activities of hydrogen peroxide." Front Plant Sci **4**: 310.
Puppo, A., K. Groten, F. Bastian, R. Carzaniga, M. Soussi, M. M. Lucas, M. R. de Felipe, J. Harrison, H. Vanacker and C. H. Foyer (2005). "Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process." New Phytologist **165**(3): 683-701.
Puppo, A., J. Rigaud and Job. D (1981). "Role of superoxide anion in leghemoglobin autooxidation." Plant Science Letters **22**: 353-360.
Ramel, F., C. Sulmon, M. Bogard, I. Couee and G. Gouesbet (2009). "Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in Arabidopsis thaliana plantlets." BMC Plant Biol **9**: 28.
Ramu, S. K., H. M. Peng and D. R. Cook (2002). "Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene rip1 in Medicago truncatula." Mol Plant Microbe Interact **15**(6): 522-528.
Raymond, J., J. L. Siefert, C. R. Staples and R. E. Blankenship (2004). "The natural history of nitrogen fixation." Mol Biol Evol **21**(3): 541-554.
Sagi, M., O. Davydov, S. Orazova, Z. Yesbergenova, R. Ophir, J. W. Stratmann and R. Fluhr (2004). "Plant respiratory burst oxidase homologs impinge on wound responsiveness and development in Lycopersicon esculentum." Plant Cell **16**(3): 616-628.
Sagi, M. and R. Fluhr (2001). "Superoxide production by plant homologues of the gp91(phox) NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection." Plant Physiology **126**(3): 1281-1290.
Sagi, M. and R. Fluhr (2006). "Production of reactive oxygen species by plant NADPH oxidases." Plant Physiol **141**(2): 336-340.
Santos, R., D. Herouart, S. Sigaud, D. Touati and A. Puppo (2001). "Oxidative burst in alfalfa-Sinorhizobium meliloti symbiotic interaction." Mol Plant Microbe Interact **14**(1): 86-89.
Sato, S., Y. Nakamura, T. Kaneko, E. Asamizu and S. Tabata (1999). "Complete structure of the chloroplast genome of Arabidopsis thaliana." DNA Res **6**(5): 283-290.
Scandalios, J. G. (2005). "Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses." Braz J Med Biol Res **38**(7): 995-1014.
Schmitt, M. P. (1997). "Utilization of host iron sources by Corynebacterium diphtheriae: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin." J Bacteriol **179**(3): 838-845.
Seki, T., M. Naruse, K. Naruse, T. Yoshimoto, A. Tanabe, M. Seki, K. Tago, T. Imaki, R. Demura and H. Demura (1999). "Induction of heme oxygenase produces load-independent cardioprotective effects in hypertensive rats." Life Sci **65**(10): 1077-1086.

Shaw, S. L. and S. R. Long (2003). "Nod factor elicits two separable calcium responses in Medicago truncatula root hair cells." Plant Physiol **131**(3): 976-984.
Sprent, J. I. (2007). "Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation." New Phytol **174**(1): 11-25.
Stocker, R., Y. Yamamoto, A. F. McDonagh, A. N. Glazer and B. N. Ames (1987). "Bilirubin is an antioxidant of possible physiological importance." Science **235**(4792): 1043-1046.
Streeter, J. G. (1981). "Effect of nitrate in the rooting medium on carbohydrate composition of soybean nodules." Plant Physiol **68**(4): 840-844.
Streeter, J. G. (1985). "Nitrate inhibition of legume nodule growth and activity : I. Long term studies with a continuous supply of nitrate." Plant Physiol **77**(2): 321-324.
Streeter, J. G. (1985). "Nitrate Inhibition of Legume Nodule Growth and Activity : II. Short Term Studies with High Nitrate Supply." Plant Physiol **77**(2): 325-328.
Sun, J., V. Cardoza, D. M. Mitchell, L. Bright, G. Oldroyd and J. M. Harris (2006). "Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation." Plant J **46**(6): 961-970.
Surpin, M., R. M. Larkin and J. Chory (2002). "Signal transduction between the chloroplast and the nucleus." Plant Cell **14 Suppl**: S327-338.
Suzuki, N., G. Miller, J. Morales, V. Shulaev, M. A. Torres and R. Mittler (2011). "Respiratory burst oxidases: the engines of ROS signaling." Curr Opin Plant Biol **14**(6): 691-699.
Tenhunen, R., H. S. Marver and R. Schmid (1968). "The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase." Proc Natl Acad Sci U S A **61**(2): 748-755.
Terry, M. J. (1997). "Phytochrome chromophore-deficient mutants." Plant Cell and Environment **20**(6): 740-745.
Terry, M. J. and R. E. Kendrick (1996). "The aurea and yellow-green-2 mutants of tomato are deficient in phytochrome chromophore synthesis." J Biol Chem **271**(35): 21681-21686.
Terry, M. J., P. J. Linley and T. Kohchi (2002). "Making light of it: the role of plant haem oxygenases in phytochrome chromophore synthesis." Biochem Soc Trans **30**(4): 604-609.
Terry, M. J., J. A. Wahleithner and J. C. Lagarias (1993). "Biosynthesis of the plant photoreceptor phytochrome." Arch Biochem Biophys **306**(1): 1-15.
Timmers, A. C., E. Soupene, M. C. Auriac, F. de Billy, J. Vasse, P. Boistard and G. Truchet (2000). "Saprophytic intracellular rhizobia in alfalfa nodules." Mol Plant Microbe Interact **13**(11): 1204-1213.
Timmis, J. N., M. A. Ayliffe, C. Y. Huang and W. Martin (2004). "Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes." Nat Rev Genet **5**(2): 123-135.
Torres, M. A., H. Onouchi, S. Hamada, C. Machida, K. E. Hammond-Kosack and J. D. Jones (1998). "Six Arabidopsis thaliana homologues of the human respiratory burst oxidase (gp91phox)." Plant J **14**(3): 365-370.
Van de Velde, W., J. C. Guerra, A. De Keyser, R. De Rycke, S. Rombauts, N. Maunoury, P. Mergaert, E. Kondorosi, M. Holsters and S. Goormachtig (2006). "Aging in legume symbiosis. A molecular view on nodule senescence in Medicago truncatula." Plant Physiol **141**(2): 711-720.

Van de Velde, W., J. C. P. Guerra, A. De Keyser, R. De Rycke, S. Rombauts, N. Maunoury, P. Mergaert, E. Kondorosi, M. Holsters and S. Goormachtig (2006). "Aging in legume symbiosis. A molecular view on nodule senescence in Medicago truncatula." Plant Physiology **141**(2): 711-720.
Vance, C. P. (2001). "Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources." Plant Physiol **127**(2): 390-397.
Vance, C. P., G. H. Heichel, D. K. Barnes, J. W. Bryan and L. E. Johnson (1979). "Nitrogen Fixation, Nodule Development, and Vegetative Regrowth of Alfalfa (Medicago sativa L.) following Harvest." Plant Physiol **64**(1): 1-8.
Vauclare, P., R. Bligny, E. Gout, V. De Meuron and F. Widmer (2010). "Metabolic and structural rearrangement during dark-induced autophagy in soybean (Glycine max L.) nodules: an electron microscopy and 31P and 13C nuclear magnetic resonance study." Planta **231**(6): 1495-1504.
Veereshlingam, H., J. G. Haynes, R. V. Penmetsa, D. R. Cook, D. J. Sherrier and R. Dickstein (2004). "nip, a symbiotic Medicago truncatula mutant that forms root nodules with aberrant infection threads and plant defense-like response." Plant Physiol **136**(3): 3692-3702.
Virtanen, A. I. and J. K. Miettinen (1963). "Biological Nitrogen Fixation " Plant Physiology, F.C. Steward (ed) Academic Press, New York **3**: 539-568.
Welinder, K. G., J. M. Mauro and L. Norskov-Lauritsen (1992). "Structure of plant and fungal peroxidases." Biochem Soc Trans **20**(2): 337-340.
Weller, J. L., M. J. Terry, C. Rameau, J. B. Reid and R. E. Kendrick (1996). "The Phytochrome-Deficient pcd1 Mutant of Pea Is Unable to Convert Heme to Biliverdin IX[alpha]." Plant Cell **8**(1): 55-67.
Willekens, H., D. Inze, M. Vanmontagu and W. Vancamp (1995). "Catalases in Plants." Molecular Breeding **1**(3): 207-228.
Wong, H. L., R. Pinontoan, K. Hayashi, R. Tabata, T. Yaeno, K. Hasegawa, C. Kojima, H. Yoshioka, K. Iba, T. Kawasaki and K. Shimamoto (2007). "Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension." Plant Cell **19**(12): 4022-4034.
Wu, M. Z., J. J. Huang, S. Xu, T. F. Ling, Y. J. Xie and W. B. Shen (2011). "Haem oxygenase delays programmed cell death in wheat aleurone layers by modulation of hydrogen peroxide metabolism." Journal of Experimental Botany **62**(1): 235-248.
Xie, Y. J., S. Xu, B. Han, M. Z. Wu, X. X. Yuan, Y. Han, Q. Gu, D. K. Xu, Q. Yang and W. B. Shen (2011). "Evidence of Arabidopsis salt acclimation induced by up-regulation of HY1 and the regulatory role of RbohD-derived reactive oxygen species synthesis." Plant J **66**(2): 280-292.
Xu, D. K., Q. J. Jin, Y. J. Xie, Y. H. Liu, Y. T. Lin, W. B. Shen and Y. J. Zhou (2011). "Characterization of a wheat heme oxygenase-1 gene and its responses to different abiotic stresses." Int J Mol Sci **12**(11): 7692-7707.
Xuan, W., S. Xu, X. Yuan and W. Shen (2008). "Carbon monoxide: A novel and pivotal signal molecule in plants?" Plant Signal Behav **3**(6): 381-382.
Yamamizo, C., K. Kuchimura, A. Kobayashi, S. Katou, K. Kawakita, J. D. Jones, N. Doke and H. Yoshioka (2006). "Rewiring mitogen-activated protein kinase cascade by positive feedback confers potato blight resistance." Plant Physiol **140**(2): 681-692.
Yannarelli, G. G., G. O. Noriega, A. Batlle and M. L. Tomaro (2006). "Heme oxygenase up-regulation in ultraviolet-B irradiated soybean plants involves reactive oxygen species." Planta **224**(5): 1154-1162.

Yendrek, C. R., Y. C. Lee, V. Morris, Y. Liang, C. I. Pislariu, G. Burkart, M. H. Meckfessel, M. Salehin, H. Kessler, H. Wessler, M. Lloyd, H. Lutton, A. Teillet, D. J. Sherrier, E. P. Journet, J. M. Harris and R. Dickstein (2010). "A putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in Medicago truncatula." Plant J **62**(1): 100-112.
Yoshioka, H., N. Numata, K. Nakajima, S. Katou, K. Kawakita, O. Rowland, J. D. Jones and N. Doke (2003). "Nicotiana benthamiana gp91phox homologs NbrbohA and NbrbohB participate in H2O2 accumulation and resistance to Phytophthora infestans." Plant Cell **15**(3): 706-718.
Yoshioka, H., K. Sugie, H. J. Park, H. Maeda, N. Tsuda, K. Kawakita and N. Doke (2001). "Induction of plant gp91 phox homolog by fungal cell wall, arachidonic acid, and salicylic acid in potato." Mol Plant Microbe Interact **14**(6): 725-736.
Zamocky, M., P. G. Furtmuller and C. Obinger (2008). "Evolution of catalases from bacteria to humans." Antioxid Redox Signal **10**(9): 1527-1548.
Zamocky, M., P. G. Furtmuller and C. Obinger (2010). "Evolution of structure and function of Class I peroxidases." Arch Biochem Biophys **500**(1): 45-57.
Zamocky, M., B. Gasselhuber, P. G. Furtmuller and C. Obinger (2012). "Molecular evolution of hydrogen peroxide degrading enzymes." Arch Biochem Biophys **525**(2): 131-144.
Zhang, C., A. Bousquet and J. M. Harris (2014). "Abscisic acid and LATD/NIP modulate root elongation via reactive oxygen species in Medicago truncatula." Plant Physiol: DOI:10.1104/pp.1114.248542.
Zhang, Y. (2014). Light regulation of legume nodulation through a plant hormone signaling network, University of Vermont.
Zilli, C. G., D. M. Santa-Cruz, G. G. Yannarelli, G. O. Noriega, M. L. Tomaro and K. B. Balestrasse (2009). "Heme Oxygenase Contributes to Alleviate Salinity Damage in Glycine max L. Leaves." Int J Cell Biol **2009**: 848516.
Zipor, G. and M. Oren-Shamir (2013). "Do vacuolar peroxidases act as plant caretakers?" Plant Sci **199-200**: 41-47.

Chapter 3: Future Directions

In this thesis I have shown that the GIRAFFE HO functions to regulate expression of ROS-regulatory genes in both seedlings and senescing nodules of *Medicago truncatula.* Such findings open up several avenues for future investigations.

Does carbon monoxide play a role in regulating ROS gene expression?

Heme Oxygenase catalyzes the degradation of heme to biliverdin with the release of carbon monoxide. Carbon monoxide acts as an important cellular signaling molecule (Xuan, Xu et al. 2008). To test if the carbon monoxide produced during heme degradation by heme oxygenase is responsible for the altered expression of ROS regulatory enzymes, *giraffe* mutants need to be exposed to carbon monoxide and their target gene expression can be compared to those of untreated *giraffe* mutants and wild-type +/- carbon monoxide. If the expression of target genes in carbon monoxide-exposed *giraffe* mutants shows a similar level as in wild-type, it can be concluded that carbon monoxide is the signaling molecule that regulates the expression of these target genes.

Does GIRAFFE HO regulate translational expression of RBOHC and PRX2?

In this study, I have shown that *GIRAFFE HO* regulates the expression of target genes transcriptionally. It would be interesting to test whether the protein expression of *RBOHC* and *cwPRX2* is also altered in the *giraffe* mutant. This would help us to understand if the regulation at the level of gene transcription is translated to protein expression as well.

What is the expression of the *RBOHC* and *PRX2* in a *gir latd* double mutant?

In this thesis I have shown that *GIRAFFE* and *LATD/NIP* oppositely regulate the expression of *RBOHC* and *cwPRX2* in six-day old seedling roots. I have also shown that *GIRAFFE* and *LATD/NIP* do not regulate expression of each other at the transcriptional level. To confirm if *GIRAFFE HO* and *LATD/NIP* act in the same pathway, we need to test the expression of their common targets in a *gir latd* double mutant. In such a double mutant, the expression of the common targets could be like that of the a) *latd* mutants b) *giraffe* mutants or c) in between. If *GIRAFFE* inhibits the function of *LATD/NIP*, i.e. *GIRAFFE* is upstream of *LATD/NIP*; the expression of the target genes in the *gir latd* double mutant would resemble that of *latd* mutant. If *LATD/NIP* inhibits the function of *GIRAFFE*, i.e. *LATD/NIP* is upstream of *GIRAFFE*; the expression of their common targets would be the same as in a *giraffe* mutant. If the expression of the common target genes matches neither with the single mutants' *giraffe* or *latd/nip*, we can confirm that the *GIRAFFE* and *LATD/NIP* regulate their common targets independent of each other and thus their effects are additive.

Do the superoxide levels or distribution pattern alter as nodules senesce? Does the *giraffe* mutation disrupt this pattern?

In this study, I have carried out ROS staining with NBT for superoxide at three time points: 25 dpi, 40 dpi and at around 70 dpi. Overall the sample size was small with as few as one nodule per genotype at some of the time points. In all samples, I found that superoxide staining was more intense in the nodule meristem. In my limited sample

size, I also noticed a decrease in the intensity of NBT stain as the nodules age. I did not notice any significant difference in superoxide staining pattern between wild-type and *giraffe* mutants. Experiments to quantify NBT stain would give conclusive evidence if there is a difference in the distribution profile of superoxide between wild-type and giraffe nodules (Ramel, Sulmon et al. 2009). The experiments will have to be designed with a greater sample size. Additionally, incorporating more time points in the experimental design and studying superoxide pattern in nitrate-induced senescence model can provide a comprehensive understanding of the distribution of superoxide during nodule senescence and whether *GIRAFFE HO* plays a role in altering the pattern. Such experiments would help find out if there are differences in superoxide distribution between a) wild-type and *giraffe* mutant nodules and b) between developmental and nitrate-induced senescence.

In which cells or tissues in the nodule is GIRAFFE HO expressed?

The regions in the nodule where GIRAFFE HO is expressed can be informative in elucidating the function and mechanism of its function. To understand which cells in the nodule express GIRAFFE HO a promoter-GUS fusion can be expressed in the nodule. Such spatio-temporal expression pattern of GIRAFFE HO will help characterize the function of GIRAFE HO.

Does *GIRAFFE HO* regulate expression of genes involved in nitrogen fixation and proteases that function in proteolytic degradation?

The efficiency of the legume-rhizobium symbiosis requires interaction between the plant and bacterial genomes. The GIRAFFE HO is expressed at high levels in a

mature nodule (*Medicago truncatula* Gene Expression Atlas (MtGEA); http://bioinfo.noble.org/gene-atlas/). It would be interesting to test whether GIRAFFE HO alters the expression of genes required for nitrogen fixation in both in the legume and the rhizobial partners. The highest expression of GIRAFFE HO is in senescing nodules (MtGEA). During nodule senescence, several proteolytic enzymes are upregulated due to various degradation pathways that are activated to bring about a programmed cell death and ensure re-mobilization of the resources in the nodule to the rest of the plant. A comparative study of the expression of these proteases between wild-type and *giraffe* mutants could give insights into the role of *GIRAFFE* HO in nodule senescence.

Does the plastid-localized HO move out of the plastid to signal the nuclear genome?

The GIRAFFE HO is a plastid-localized enzyme that serves as a molecule of communication in the process of chloroplast-nucleus signaling (Surpin, Larkin et al. 2002). Such a signaling mechanism ensures the proper development and functioning of the chloroplast and possibly regulates other cellular functions (Nott, Jung et al. 2006). The question arises, as part of the signaling mechanism; does the HO itself leave the chloroplast and move to the nucleus to regulate transcription of the nuclear genome? Although this seems unlikely, in fact this is the fate of the animal HO1 which has been shown to move from the endoplasmic reticulum to the mitochondria and the nucleus (Lin, Weis et al. 2007, Bansal, Biswas et al. 2014). The movement of the MtHO within the cell can be monitored by expressing a HO-GFP fusion protein and observing it on a confocal microscope. The answer to the mobility of the HO can

give clues to the mechanism by which GIRAFFE HO regulates nuclear gene expression.

Is HO plastid-localized in the root?

Heme Oxygenase is localized to the chloroplast, a differentiated plastid present in the leaves and stems of plants (Muramoto, Kohchi et al. 1999). We do not know whether plastids that are present in roots contain heme oxygenase. To test this, the localization of the GIRAFFE HO-GFP fusion protein inside a root cell should be investigated. If the fusion protein is detected in the plastid, it means that HO is localized in the plastids of root.

The experiments described here would help further characterize the expression and the regulation of *GIRAFFE* HO. This would also help to understand the functions of the only known heme oxygenase gene in the *Medicago truncatula* genome.

References

Bansal, S., G. Biswas and N. G. Avadhani (2014). "Mitochondria-targeted heme oxygenase-1 induces oxidative stress and mitochondrial dysfunction in macrophages, kidney fibroblasts and in chronic alcohol hepatotoxicity." Redox Biol **2**: 273-283.
Lin, Q., S. Weis, G. Yang, Y. H. Weng, R. Helston, K. Rish, A. Smith, J. Bordner, T. Polte, F. Gaunitz and P. A. Dennery (2007). "Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress." J Biol Chem **282**(28): 20621-20633.
Muramoto, T., T. Kohchi, A. Yokota, I. Hwang and H. M. Goodman (1999). "The Arabidopsis photomorphogenic mutant hy1 is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase." Plant Cell **11**(3): 335-348.
Nott, A., H. S. Jung, S. Koussevitzky and J. Chory (2006). "Plastid-to-nucleus retrograde signaling." Annu Rev Plant Biol **57**: 739-759.
Ramel, F., C. Sulmon, M. Bogard, I. Couee and G. Gouesbet (2009). "Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in Arabidopsis thaliana plantlets." BMC Plant Biol **9**: 28.

Surpin, M., R. M. Larkin and J. Chory (2002). "Signal transduction between the chloroplast and the nucleus." Plant Cell **14 Suppl**: S327-338.
Xuan, W., S. Xu, X. Yuan and W. Shen (2008). "Carbon monoxide: A novel and pivotal signal molecule in plants?" Plant Signal Behav **3**(6): 381-382.