Transcriptional regulation of nodule development and senescence in *Medicago truncatula*

Vidyadhar Karmarkar

Thesis committee

Promotor

Prof. Dr Ton Bisseling
Professor of Molecular Biology (Development Biology of Plants)
Wageningen University

Co-promotor
Dr Rene Geurts
Assistant professor, Laboratory of Molecular Biology
Wageningen University

Other members
Prof. Dr Richard Immink, Wageningen University
Dr Wouter Kohlen, Max-Planck Institute for Plant Breeding, Köln, Germany
Dr Leo van Overbeek, Wageningen University
Dr Liesje Mommer, Wageningen University

This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

Transcriptional regulation of nodule development and senescence in *Medicago truncatula*

Vidyadhar Karmarkar

Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 19 May 2014
at 11 a.m. in the Aula.

Vidyadhar Karmarkar
Transcriptional regulation of nodule development and senescence in *Medicago truncatula*, 110 pages.

PhD thesis, Wageningen University, Wageningen, NL (2014)
With references, with summaries in Dutch and English

ISBN 978-94-6257-021-4

CONTENTS

Outline

In this thesis, I describe the role of two transcription factors – *MtNAC074* and *MtNAC920* - of the NAC (NAM, ATAF, CUC) family that control symbiotic nitrogen fixation in the model legume *Medicago truncatula* in response to exogenous soil nitrate. Soil nitrates are known to inhibit symbiotic nitrogen fixation by affecting nodule initiation and development and by triggering premature nodule senescence. Although this inhibitory effect of nitrate is known for almost a century, the underlying molecular mechanisms remain unknown. The characterization of *MtNAC074* and *MtNAC920* presented in this thesis provides first insights to the signaling pathways that control the senescence response in nitrogen fixing root nodules.

Transcription factors are central to gene regulation as these proteins have affinity to certain *cis*-elements in the genome to which they can bind in a sequence specific manner. Transcription factors are of agricultural interest because they regulate diverse plant functions ranging from developmental processes to adaptability to the environment. Hence, determining the repertoire of transcription factors in a genome, the regulation of their expression, their protein interactors and their direct targets are important for understanding the gene regulatory networks and evolution. While the model plant *Arabidopsis thaliana* has been immensely useful in understanding the role of transcription factors in shaping plant biological processes common to higher plants, it lacks certain traits that are of agricultural interest. Among these traits are plant symbiotic interactions with nitrogen-fixing bacteria or nutrient scavenging mycorrhizal fungi. By the development of model legumes, like *M. truncatula*, it is now possible to untangle the role of transcription factors that control these agricultural important symbiotic traits.

M. truncatula genome encodes more than 3,000 putative transcription factors [3]. However, less than 1% has been functionally characterized [3]. For functional characterization of *M. truncatula* transcription factors, several community resources are available including a high-throughput qRT-PCR platform, a gene expression atlas based an Affymetrix GeneChip and a TNT1 transposon tagged mutant population [4]. Besides these community platforms, RNA-seq can be employed as a more targeted approach to test specific hypotheses. RNA-Seq is a very powerful approach for identifying differentially expressed as well as alternatively spliced transcripts because it is more sensitive than microarrays [12, 13]

Using these community resources (**Chapter 2**) as well as by employing RNA-seq (**Chapter 4**), I discovered two NAC transcription factors, *MtNAC074* and *MtNAC920,* that are upregulated in *M. truncatula* nodules within several hours after application of 15 mM NH_4NO_3 to the root. The nitrate-dependent upregulation of both transcription factors is nodule specific and requires ethylene biosynthesis and signaling. Chemical interference in ethylene biosynthesis and genetic attenuation of ethylene signaling hindered the nitrate-dependent upregulation of these transcription factors. Importantly, the ectopic expression of *MtNAC074* and *MtNAC920* in the root nodule affected symbiosome development in case of *MtNAC074* (**Chapter 3**) and symbiosome integrity leading to senescence in case of *MtNAC920* (**Chapter 5**). MtNAC074 directly bound to the promoter region of the members of vesicle associated membrane protein (VAMP) family, *MtVAMP721* (**Chapter 3**). On the other hand, MtNAC920 directly bound to the promoter region of a cysteine protease, *MtCP2*. *MtVAMP721* genes were shown to be important for exocytotic delivery of cargo essential for symbiosome formation [1]. The overexpression of *MtCP2* triggered nodule senescence (**Chapter 5**) whereas the silencing of its homolog delayed nodule senescence in *Astragalus sinicus* [2]. The ectopic expression of *MtNAC074* and *MtNAC920* led to the downregulation of *MtVAMP721* and *MtCP2* respectively. These data suggest that MtNAC074 and MtNAC920 may act as repressors of *MtVAMP721* and *MtCP2* expression. Based on this, I propose a model that explains how exogenous nitrate may affect symbiotic nitrogen fixation (Figure 1) (**Chapter 6**). According to this model, SKL/EIN2 acts as a molecular hub that connects nitrate-derived ethylene signals to the downstream transcriptional response. Through *SKL/EIN2*, nitrate may activate *MtNAC074* or *MtNAC920* to affect nodule development or trigger nodule senescence respectively.

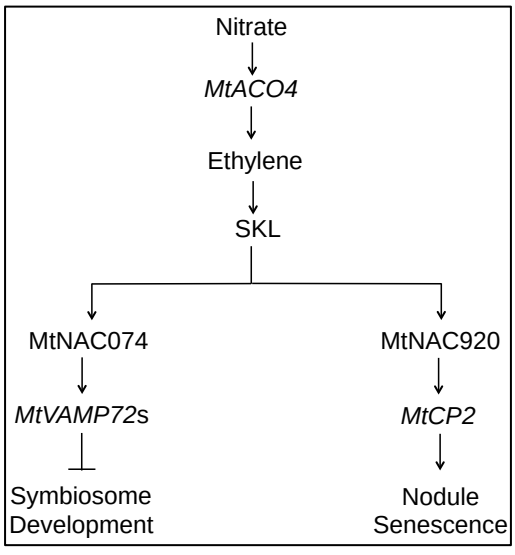

Figure 1 A conceptual model depicting nitrate induced signal transduction cascade leading to decreased symbiotic nitrogen fixation.

Ethylene is deployed in plant stress pathways as well as in development [18]. In nodule organogenesis, ethylene was shown to play an important role in regulating nodule number [19-22]. The ethylene signaling mutant, *skl*, was shown to be a hypernodulator because of persistent rhizobial infections. Likewise, ethylene was also shown to be release in legume roots and nodules upon nitrate application [23, 24]. Given these data, a

plausible and yet untested hypothesis was ethylene is a secondary messenger induced by exogenous soil nitrate. Herein, I demonstrated that ethylene might act as a signal to regulate symbiosome development and senescence. Thus, ethylene is an important hormone required for birth and death of a nitrogen-fixing root nodule.

Outlook

The molecular basis of nodule senescence was not well understood. My research has identified important molecular nodes required to lay a conceptual framework of the process. As with any research, the current study has raised several questions that lay the foundation for future studies. For instance, it will be of importance to study the nodule phenotype of *Mtnac074* knock out lines to assess whether this gene is a key responsive regulator respect to exogenous nitrate. Likewise, studies using knockout lines of *Mtcp2* and other cysteine proteases are important to study nodule longevity. Furthermore, for detailed subcellular characterization of overexpression and silencing phenotypes, transmission electron microscopy is indispensible component of any study on nodule development and senescence. Lastly, I propose, instead of using strong promoters like *Ubiquitin* (this study) or CaMV35S, to use cell-type specific promoters or an inducible system for gene activation for further functional characterization of genes in nodule development and senescence.

References

1. Ivanov S, Fedorova EE, Limpens E, De Mita S, Genre A, Bonfante P, Bisseling T: **Rhizobium-legume symbiosis shares an exocytotic pathway required for arbuscule formation**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(21):8316-8321.
2. Naito Y, Fujie M, Usami S, Murooka Y, Yamada T: **The involvement of a cysteine proteinase in the nodule development in Chinese milk vetch infected with Mesorhizobium huakuii subsp. rengei**. *Plant physiology* 2000, **124**(3):1087-1096.
3. Udvardi MK, Kakar K, Wandrey M, Montanari O, Murray J, Andriankaja A, Zhang JY, Benedito V, Hofer JM, Chueng F *et al*: **Legume transcription factors: global regulators of plant development and response to the environment**. *Plant physiology* 2007, **144**(2):538-549.
4. Kakar K, Wandrey M, Czechowski T, Gaertner T, Scheible WR, Stitt M, Torres-Jerez I, Xiao Y, Redman JC, Wu HC *et al*: **A community resource for high-throughput quantitative RT-PCR analysis of transcription factor gene expression in Medicago truncatula**. *Plant methods* 2008, **4**:18.
5. Gruber V, Blanchet S, Diet A, Zahaf O, Boualem A, Kakar K, Alunni B, Udvardi M, Frugier F, Crespi M: **Identification of transcription factors involved in root apex responses to salt stress in Medicago truncatula**. *Molecular genetics and genomics* 2009, **281**(1):55-66.
6. de Zélicourt A, Diet A, Marion J, Laffont C, Ariel F, Moison M, Zahaf O, Crespi M, Gruber V, Frugier F: **Dual involvement of a Medicago truncatula NAC transcription factor in root abiotic stress response and symbiotic nodule senescence**. *The Plant Journal* 2012, **70**(2):220-230.
7. Ariel F, Diet A, Verdenaud M, Gruber V, Frugier F, Chan R, Crespi M: **Environmental regulation of lateral root emergence in Medicago truncatula requires the HD-Zip I transcription factor HB1**. *The Plant cell* 2010, **22**(7):2171-2183.
8. He J, Benedito VA, Wang M, Murray JD, Zhao PX, Tang Y, Udvardi MK: **The Medicago truncatula gene expression atlas web server**. *BMC bioinformatics* 2009, **10**:441.
9. Verdier J, Zhao J, Torres-Jerez I, Ge S, Liu C, He X, Mysore KS, Dixon RA, Udvardi MK: **MtPAR MYB transcription factor acts as an on switch for proanthocyanidin biosynthesis in Medicago truncatula**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(5):1766-1771.
10. Sinharoy S, Torres-Jerez I, Bandyopadhyay K, Kereszt A, Pislariu CI, Nakashima J, Benedito VA, Kondorosi E, Udvardi MK: **The C2H2 Transcription Factor REGULATOR OF SYMBIOSOME DIFFERENTIATION Represses Transcription of the Secretory Pathway Gene VAMP721a and Promotes Symbiosome Development in Medicago truncatula**. *The Plant cell* 2013, **25**(9):3584-3601.
11. Tayeh N, Bahrman N, Sellier H, Bluteau A, Blassiau C, Fourment J, Bellec A, Debelle F, Lejeune-Henaut I, Delbreil B: **A tandem array of CBF/DREB1 genes is located in a major freezing tolerance QTL region on Medicago truncatula chromosome 6**. *BMC genomics* 2013, **14**(1):814.
12. Wang Z, Gerstein M, Snyder M: **RNA-Seq: a revolutionary tool for transcriptomics**. *Nature reviews Genetics* 2009, **10**(1):57-63.
13. Ozsolak F, Milos PM: **RNA sequencing: advances, challenges and opportunities**. *Nature reviews Genetics* 2011, **12**(2):87-98.
14. Severin AJ, Woody JL, Bolon YT, Joseph B, Diers BW, Farmer AD, Muehlbauer GJ, Nelson RT, Grant D, Specht JE *et al*: **RNA-Seq Atlas of Glycine max: a guide to the soybean transcriptome**. *BMC plant biology* 2010, **10**:160.

15. Boscari A, Del Giudice J, Ferrarini A, Venturini L, Zaffini AL, Delledonne M, Puppo A: **Expression dynamics of the Medicago truncatula transcriptome during the symbiotic interaction with Sinorhizobium meliloti: which role for nitric oxide?** *Plant physiology* 2013, **161**(1):425-439.
16. Moreau S, Verdenaud M, Ott T, Letort S, de Billy F, Niebel A, Gouzy J, de Carvalho-Niebel F, Gamas P: **Transcription Reprogramming during Root Nodule Development in *Medicago truncatula***. *PloS one* 2011, **6**(1):e16463.
17. Guo Y, Cai Z, Gan S: **Transcriptome of Arabidopsis leaf senescence**. *Plant, cell & environment* 2004, **27**(5):521-549.
18. Ecker: **The ethylene signal transduction pathway in plants**. *Science* 1995, **268**(5211):667-675.
19. Sun J, Cardoza V, Mitchell DM, Bright L, Oldroyd G, Harris JM: **Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation**. *The Plant journal : for cell and molecular biology* 2006, **46**(6):961-970.
20. Prayitno J, Rolfe BG, Mathesius U: **The Ethylene-insensitive sickle mutant of Medicago truncatula shows altered auxin transport regulation during nodulation**. *Plant physiology* 2006, **142**(1):168-180.
21. Penmetsa RV, Uribe P, Anderson J, Lichtenzveig J, Gish JC, Nam YW, Engstrom E, Xu K, Sckisel G, Pereira M *et al*: **The Medicago truncatula ortholog of Arabidopsis EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations**. *The Plant journal* 2008, **55**(4):580-595.
22. Penmetsa RV, Cook DR: **A Legume Ethylene-Insensitive Mutant Hyperinfected by Its Rhizobial Symbiont**. *Science* 1997, **275**(5299):527-530.
23. Caba JM, Recalde L, Ligero F: **Nitrate-induced ethylene biosynthesis and the control of nodulation in alfalfa**. *Plant, cell & environment* 1998, **21**(1):87-93.
24. Ligero F, Caba JM, Lluch C, Olivares J: **Nitrate Inhibition of Nodulation Can Be Overcome by the Ethylene Inhibitor Aminoethoxyvinylglycine**. *Plant physiology* 1991, **97**(3):1221-1225.

Chapter 1

Symbiotic Nitrogen Fixation to Mitigate Climate Change

Author: Vidyadhar Karmarkar[1,§]

Affiliations:

1. Laboratory of Molecular Biology, Department of Plant Science, Wageningen

FOOTNOTE:

§ Current Address: Max Planck Institute for Plant Breeding,
Carl-von-Linne-Weg 10, 50829 Cologne, Germany.

Surplus anthropogenic nitrogen

Early 20th century two German scientists – Fritz Haber and Carl Bosch- invented a method that at high temperature and pressure produces ammonia by a chemical reaction of nitrogen and hydrogen gas; a method that became known as the Haber-Bosch process. This Haber-Bosch nitrogen literally changed the world. While catalyzing the slaughter of millions in armed conflict with its use in explosives, it also gave life to billions through nitrogen fertilizers. It is estimated that nitrogen fertilizers consume 80% of the Haber-Bosch nitrogen [1]. Nevertheless, a large proportion of this anthropogenic nitrogen is lost to the environment due to the low nitrogen use efficiency of cultivated crops. For instance, in 2005, 100 Tg of the Haber-Bosch nitrogen was used in global agriculture, but only 17 Tg nitrogen was consumed by mankind through crops, dairy or meat products [2]. The unutilized soil nitrogen is highly mobile and triggers a cascade of environmental problems. For instance, the residual nitrogen is converted to various kinds of nitrogen containing gases e.g. nitrous oxide (N_2O). N_2O contributes to climate forcing [3] and has been identified as the major ozone-depleting substance in the 21st century [4]. In addition, the reactive nitrogen results in eutrophication, algal bloom, hypoxia along the coastal ocean [5], loss of terrestrial and marine biodiversity, invasive weed growth, acid rains, increase in soil acidity and affects carbon storage in peatlands and contaminates drinking water [6]. Another aspect of environmental damage is the carbon footprint of current methods to manufacture ammonia. It is estimated that for every ton of ammonia produced, 1.87 tons of CO_2 is released [7]. This implied in 2012, for the 137 million tons of global ammonia production [8], 256.19 million tons of CO_2 was released. CO_2 is a potent greenhouse gas and a significant contributor to global warming. Taken together, the anthropogenic fixed nitrogen is one of the major drivers of climate change.

To put this environmental damage in an economic perspective, for the year 2008, the annual cost of agriculture-derived surplus anthropogenic nitrogen just to the European Union was estimated to be between €40 billion and €230 billion [6]. After factoring the losses from non-agricultural sources, the total environmental cost inflates to €75 billion and €485 billion. This is equivalent to €150-€1150 per European citizen and 2% of the Gross Domestic Product [9]. Similarly, the potential agricultural income that was lost due to the unutilized fertilizer and manure was between €13 billion and €16 billion per year [6]. In addition to the economic loss, the unutilized fertilizer also impacts the global energy budget. Based on a recent study, 40% of the fertilizer nitrogen that is lost to the environment is denitrified to the inert

atmospheric nitrogen [6]. Although this loss to the environment is innocuous, it represents a waste of the energy invested in Haber-Bosch process. This energy loss is equivalent to 32 MJ kg^{-1} of fixed nitrogen or approximately 1% of global primary energy supply [2, 10]. Hence, there is an urgent need to mitigate the detrimental environmental and economic impact of anthropogenic nitrogen.

To curb further agro-ecological degradation by anthropogenic nitrogen, the amended Gothenburg Protocol sets ceilings on the emission of ammonia and other pollutants [11]. In addition to the emission restrictions, a multi-pronged integrated nitrogen management strategy is critical to mitigate climate change caused by anthropogenic nitrogen has to be applied. Such a robust integrated nitrogen management strategy should include following aspects:

1. Precision agriculture [12-14]: 50-70% of nitrogen fertilizer is wasted because of excess use of artificial fertilizers and poor nitrogen uptake and assimilation by the plant. Hence, it is necessary to optimize the rate, time and method of fertilizer application.
2. Nitrogen Use Efficiency (NUE) [15]: The NUE of plants is dependent on the uptake fixed nitrogen from the soil and the subsequent utilization efficiency. The latter trait can be further compartmentalized into assimilation and translocation/remobilization efficiency. Because, nitrogen uptake, assimilation and translocation are genetically controlled traits [16], it should be possible to genetically manipulate plants for improved the nitrogen economy.
3. Legume-based cropping systems [17]: Deploying legume-based cropping systems to fulfill the nitrogen requirement of agriculture.
4. Industrial Engineering [18]: Developing processes with reduced carbon footprint to manufacture nitrogen fertilizers.
5. Environmental policy [11]: While policy measures like Gothenburg protocol sets the limits on emissions, more restrictive measures should be adopted by each country e.g. issuing fertilizers based on need (as determined by rate, time, method).

Here, I describe the benefits of legume-based cropping systems as a potent measure to mitigate climate change. Legume cultivation is regaining attention as a sustainable nitrogen source for agriculture [17, 19, 20]. This renewed interest stems from the natural ability of legumes of symbiotic nitrogen fixation, which is estimated to be 40 Tg per year [1]. However,

the projected global nitrogen fertilizer requirement by the year 2100 ranges between 100-200 Tg N [2]. This implies the “gap” in symbiotic nitrogen fixation by the year 2100 ranges from 60-160 Tg N. Thus, there is an enormous opportunity for bridging this gap through genetic improvement of symbiotic nitrogen fixation and better agronomic practices. Herein, we review the current research to address the question whether symbiotic nitrogen fixation can mitigate climate change.

Legume-based cropping systems

In legume-based cropping systems, symbiotic nitrogen fixation can fulfill the nitrogen requirement of the host legume for seed production and biomass as well as companion and rotation crops through rhizodepostion [21, 22]. More importantly, symbiotic nitrogen fixation is free, renewable, and sustainable and provides a significant environmental and economic “comparative advantage” over nitrogen fertilizers. Apart from the comparative advantage of symbiotic nitrogen fixation, legume-based cropping systems also have following benefits – (i) legume-cereal rotation or intercrop enhances yield, reduces incidence of root rot and foliar diseases and pests [23, 24] implying net savings by reducing the input costs. (ii) Legume-cereal rotations have improved energy economy by decreasing the average energy usage by 12-34%. (iii) Legume crops and pastures use 35-60% less fossil energy compared to nitrogen-fertilized cereals or non-legume pastures [17]. (iv) Legume crops are instrumental in decreasing the carbon footprint of agriculture (measured in carbon dioxide equivalence or CO_2e). Legume crops produce 0.2 to 0.33 kg CO_2e per kilogram of grain product as compared to 0.46-0.80 kg CO_2e for cereals and oilseeds. Similarly, a legume-cereal rotation emit 20% lower CO_2e as compared to a cereal-cereal rotation [25]. Thus, legume-based cropping system is an economically and environmentally prudent strategy to meet the agricultural nitrogen requirement while mitigating climate change.

Legume Breeding for Symbiotic Nitrogen Fixation

Legumes gain their ability from symbiotic nitrogen fixation from their symbiotic association with the cognate rhizobial bacteria. The biology of initiation and establishment of the symbiosis has been intense fundamental research [26]. Because of my interest in the agricultural context of legume biology, I will only touch those biological concepts that will be important in building current thesis. Instead, I will briefly review recent literature on breeding and biotechnology approaches to improve symbiotic nitrogen fixation.

The symbiosis begins with an intimate molecular dialogue between the host legume and invading rhizobia. The roots of a host release flavonoid signals, which signal the cognate rhizobia to produce lipochitooligosaccharide compounds called the Nod factors. The Nod factors trigger multiple responses in the host that facilitate bacterial invasion. One of such initial responses resulting from a successful host-rhizobial recognition is curling of the root hair. The curled root hair entraps the rhizobia attached to the surface of a root hair. Such entrapped rhizobia start multiplying to form an infection thread that penetrates the root. Simultaneously, mitotic division is initiated in the root cortical cells, which internalize the rhizobia from the infection thread. During the process of internalization, the rhizobia are engulfed in the plant membrane and gain a new identity called the symbiosome. These intracellular rhizobia differentiate into bacteroids, which are the fundamental unit of symbiotic nitrogen fixation found in nature. The net result of this process is the birth of a new root organ called the nodule [27, 28].

Based on the activity of nodule meristem, nodules can be classified as determinate and indeterminate. Determinate nodules are characterized by a persistent meristem e.g. *Lotus japonicus* and *Glycine max* (soybean). Indeterminate nodules are round and characterized by non-persistent meristem e.g. *Medicago truncatula* and *Pisum sativum* (pea). The indeterminate activity of a non-persistent meristem results in a cylindrical nodule that can be divided into four zones – meristem, infection, fixation and senescence. In indeterminate nodules, the senescence develops along the longitudinal axis whereas in determinate nodules the senescence zone radiates from the central tissue. The senescence zone is absent in a young nodule, but develops with nodule age or on exposure to abiotic stress [29] e.g. drought [30], salt [31] and darkness [32]. Nodule senescence is genetically controlled process [29] and has been proposed to be conceptually similar to leaf senescence [33].

Despite the advances in the understanding of the molecular biology and genetics of nodule development and senescence, legume breeding for enhanced symbiotic nitrogen fixation has met limited success [34, 35]. This is surprising because, on one hand, the notion of breeding for enhancing symbiotic nitrogen fixation has been prevalent for decades [36]. On the other hand, traits (e.g. abiotic and biotic stress) other than symbiotic nitrogen fixation have been a priority in legume breeding programs [37]. Perhaps, the lack of success is because of the complexity of the genetics of symbiotic nitrogen fixation, which requires spatiotemporal coordination of the host as well as rhizobial gene expression [27]. Breeding

for coordinating host-symbiont interaction is not a trivial task. Notwithstanding the odds, because of the availability of resources for legume genomics and breeding [37], genetic improvement of symbiotic nitrogen fixation is regaining priority. For example, quantitative trait loci (QTL) analysis in *M. truncatula* recombinant populations identified two epistatic interactions regulating symbiotic nitrogen fixation [38]. Furthermore, symbiotic nitrogen fixation can also be improved by selecting for associated traits like nodule number [39], shoot dry weight [40] and plant growth habit [41]. In *Phaseolus vulgaris* (common bean), the analysis of a recombinant inbred line (RIL) population identified QTLs for nitrogen fixation, nitrogen content at late pod-filling stage and at harvest [41]. Similarly, in *L. japonicus*, co-localization of QTLs underlying symbiotic nitrogen fixation and seed mass was observed [42]. Because exogenous nitrogen application at grain-filling stage improved yield and protein content of seeds [43], colocalization of nitrogen fixation and pod-filling/seed mass related QTLs could potentially harbor genes responsible for enhanced yield. Such QTLs are important for sustainable agriculture as it could meet the long sought goal of deploying symbiotic nitrogen fixation to increase legume yield. Because symbiotic nitrogen fixation declines with abiotic stress, breeding for abiotic stress tolerance could also potentially generate cultivars with enhanced symbiotic nitrogen fixation. For instance, a QTL for saline [44] as well as alkaline [45] tolerance has been identified in soybean. In another study, using a restriction site polymorphism-based candidate gene mapping strategy, genes responsible for drought tolerance in *Vigna ungiculata* were identified [46]. However, it remains to be verified if the QTLs and candidate genes associated with stress tolerance also enhance nitrogen fixation. Notwithstanding these studies, alike other traits of biotic/abiotic stress, yield, oil content etc, symbiotic nitrogen fixation as a trait needs more attention from the scientific community.

Concluding Remarks

In conclusion, given the urgent need to develop crops that while mitigating climate change facilitate achieving food security, more research on nitrogen fixation is indispensible. The legume genomics resources [37, 47, 48] provide unprecedented opportunities for genomics-assisted breeding to enhance nitrogen fixation. The micro- and macro-synteny between legume genomes [49] also provides an additional leverage for translational genomics [50, 51]. Such leverage allows access to legumes other than soybean thus increasing the repertoire of crops that can be deployed for food security while mitigating climate change. I believe that the recent access to genomic information, advances in technology like high

throughput sequencing should allow us to enhance symbiotic nitrogen fixation to an extent that it is agriculturally successful and sustainable strategy to mitigate climate change. Hence, in this thesis, I have attempted to unravel transcriptional control of symbiotic nitrogen fixation. The methods and discoveries described herein can be potentially used for genetic improvement of symbiotic nitrogen fixation.

References

1. Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA: **Transformation of the nitrogen cycle: recent trends, questions, and potential solutions**. *Science* 2008, **320**(5878):889-892.
2. Erisman JW, Sutton MA, Galloway J, Klimont Z, Winiwarter W: **How a century of ammonia synthesis changed the world**. *Nature Geosci* 2008, **1**(10):636-639.
3. Robertson GP, Vitousek PM: **Nitrogen in Agriculture: Balancing the Cost of an Essential Resource**. *Annual Review of Environment and Resources* 2009, **34**(1):97-125.
4. Ravishankara AR, Daniel JS, Portmann RW: **Nitrous oxide (N2O): the dominant ozone-depleting substance emitted in the 21st century**. *Science* 2009, **326**(5949):123-125.
5. Diaz RJ, Rosenberg R: **Spreading dead zones and consequences for marine ecosystems**. *Science* 2008, **321**(5891):926-929.
6. Sutton MA, Oenema O, Erisman JW, Leip A, van Grinsven H, Winiwarter W: **Too much of a good thing**. *Nature* 2011, **472**(7342):159-161.
7. http://www.kbr.com/Newsroom/Publications/Articles/Carbon-Dioxide- Capture-and-Storage-in-the-Nitrogen-Syngas-Industries.pdf. (Access 28th August. In.
8. http://minerals.usgs.gov/minerals/pubs/commodity/nitrogen/mcs-2013-nitro.pdf.
9. van Grinsven HJ, Holland M, Jacobsen BH, Klimont Z, Sutton MA, Willems WJ: **Costs and benefits of nitrogen for Europe and implications for mitigation**. *Environmental science & technology* 2013.
10. Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green PA, Holland EA *et al*: **Nitrogen Cycles: Past, Present, and Future**. *Biogeochemistry* 2004, **70**(2):153-226.
11. http://www.unece.org/env/lrtap/multi_h1.html.
12. Gebbers R, Adamchuk VI: **Precision agriculture and food security**. *Science* 2010, **327**(5967):828-831.
13. Pierce FJ, Nowak P: **Aspects of Precision Agriculture**. 1999, **67**:1-85.
14. Diacono M, Rubino P, Montemurro F: **Precision nitrogen management of wheat. A review**. *Agronomy for Sustainable Development* 2012, **33**(1):219-241.
15. McAllister CH, Beatty PH, Good AG: **Engineering nitrogen use efficient crop plants: the current status**. *Plant biotechnology journal* 2012, **10**(9):1011-1025.
16. Gutierrez RA: **Systems biology for enhanced plant nitrogen nutrition**. *Science* 2012, **336**(6089):1673-1675.
17. Jensen ES, Peoples MB, Boddey RM, Gresshoff PM, Hauggaard-Nielsen H, Alves B, Morrison MJ: **Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review**. *Agronomy for Sustainable Development* 2011, **32**(2):329-364.
18. Lan R, Irvine JT, Tao S: **Synthesis of ammonia directly from air and water at ambient temperature and pressure**. *Scientific reports* 2013, **3**:1145.
19. Jensen E, Hauggaard-Nielsen H: **How can increased use of biological N2 fixation in agriculture benefit the environment?** *Plant and Soil* 2003, **252**(1):177-186.
20. Drinkwater LE, Wagoner P, Sarrantonio M: **Legume-based cropping systems have reduced carbon and nitrogen losses**. *Nature* 1998, **396**(6708):262-265.
21. Fustec J, Lesuffleur F, Mahieu S, Cliquet J-B: **Nitrogen rhizodeposition of legumes. A review**. *Agronomy for Sustainable Development* 2010, **30**(1):57-66.
22. Peoples MB, Herridge DF, Ladha JK: **Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production?** *Plant and Soil* 1995, **174**(1-2):3-28.

23. Danga BO, Ouma JP, Wakindiki IIC, Bar-Tal A: **Chapter 5 Legume–Wheat Rotation Effects on Residual Soil Moisture, Nitrogen and Wheat Yield in Tropical Regions**. 2009, **101**:315-349.
24. Hauggaard-Nielsen H, Jørnsgaard B, Kinane J, Jensen ES: **Grain legume–cereal intercropping: The practical application of diversity, competition and facilitation in arable and organic cropping systems**. *Renewable Agriculture and Food Systems* 2008, **23**(01).
25. Gan Y, Liang C, Hamel C, Cutforth H, Wang H: **Strategies for reducing the carbon footprint of field crops for semiarid areas. A review**. *Agronomy for Sustainable Development* 2011, **31**(4):643-656.
26. Oldroyd GED, Murray JD, Poole PS, Downie JA: **The Rules of Engagement in the Legume-Rhizobial Symbiosis**. *Annual Review Genetics, Vol 45* 2011, **45**:119-144.
27. Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC: **How rhizobial symbionts invade plants: the Sinorhizobium-Medicago model**. *Nature reviews Microbiology* 2007, **5**(8):619-633.
28. Oldroyd GE, Downie JA: **Coordinating nodule morphogenesis with rhizobial infection in legumes**. *Annual review of plant biology* 2008, **59**:519-546.
29. Puppo A, Groten K, Bastian F, Carzaniga R, Soussi M, Lucas MM, de Felipe MR, Harrison J, Vanacker H, Foyer CH: **Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process**. *The New phytologist* 2005, **165**(3):683-701.
30. Larrainzar E, Wienkoop S, Scherling C, Kempa S, Ladrera R, Arrese-Igor C, Weckwerth W, González EM: **Carbon Metabolism and Bacteroid Functioning Are Involved in the Regulation of Nitrogen Fixation in Medicago truncatula Under Drought and Recovery**. *Molecular Plant-Microbe Interactions* 2009, **22**(12):1565-1576.
31. Bekki A, Trinchant J-C, Rigaud J: **Nitrogen fixation (C2H2 reduction) by Medicago nodules and bacteroids under sodium chloride stress**. *Physiologia plantarum* 1987, **71**(1):61-67.
32. Gogorcena Y, Gordon AJ, Escuredo PR, Minchin FR, Witty JF, Moran JF, Becana M: **N2 Fixation, Carbon Metabolism, and Oxidative Damage in Nodules of Dark-Stressed Common Bean Plants**. *Plant physiology* 1997, **113**(4):1193-1201.
33. Van de Velde W, Guerra JC, De Keyser A, De Rycke R, Rombauts S, Maunoury N, Mergaert P, Kondorosi E, Holsters M, Goormachtig S: **Aging in legume symbiosis. A molecular view on nodule senescence in Medicago truncatula**. *Plant physiology* 2006, **141**(2):711-720.
34. Herridge D, Rose I: **Breeding for enhanced nitrogen fixation in crop legumes**. *Field Crops Research* 2000, **65**(2–3):229-248.
35. Shantharam S, Mattoo A: **Enhancing biological nitrogen fixation: An appraisal of current and alternative technologies for N input into plants**. *Plant and Soil* 1997, **194**(1-2):205-216.
36. LaRue TA: **Selecting and breeding legumes for enhanced nitrogen fixation.** *In:Proceedings of a Workshop, Boyce Thompson Institute, Cornell, 1978, 23pp* 1978.
37. Varshney RK, Thundi M, May GD, Jackson SA: **Legume Genomics and Breeding**. In: *Plant Breeding Reviews.* John Wiley & Sons, Inc.; 2010: 257-304.
38. Moreau D, Burstin J, Aubert G, Huguet T, Ben C, Prosperi JM, Salon C, Munier-Jolain N: **Using a physiological framework for improving the detection of quantitative trait loci related to nitrogen nutrition in Medicago truncatula**. *TAG Theoretical and applied genetics Theoretische und angewandte Genetik* 2012, **124**(4):755-768.

39. Nicolás MF, Hungria M, Arias CAA: **Identification of quantitative trait loci controlling nodulation and shoot mass in progenies from two Brazilian soybean cultivars**. *Field Crops Research* 2006, **95**(2-3):355-366.
40. Bourion V, Rizvi SM, Fournier S, de Larambergue H, Galmiche F, Marget P, Duc G, Burstin J: **Genetic dissection of nitrogen nutrition in pea through a QTL approach of root, nodule, and shoot variability**. *TAG Theoretical and applied genetics Theoretische und angewandte Genetik* 2010, **121**(1):71-86.
41. Ramaekers L, Galeano C, Garzón N, Vanderleyden J, Blair M: **Identifying quantitative trait loci for symbiotic nitrogen fixation capacity and related traits in common bean**. *Mol Breeding* 2013, **31**(1):163-180.
42. Tominaga A, Gondo T, Akashi R, Zheng S-h, Arima S, Suzuki A: **Quantitative trait locus analysis of symbiotic nitrogen fixation activity in the model legume Lotus japonicus**. *J Plant Res* 2012, **125**(3):395-406.
43. Salon C, Munier-Jolain NG, Duc G, Voisin A-S, Grandgirard D, Larmure A, Emery RJN, Ney B: **Grain legume seed filling in relation to nitrogen acquisition: A review and prospects with particular reference to pea**. *Agronomie* 2001, **21**(6-7):539-552.
44. Lee GJ, Carter TE, Jr., Villagarcia MR, Li Z, Zhou X, Gibbs MO, Boerma HR: **A major QTL conditioning salt tolerance in S-100 soybean and descendent cultivars**. *TAG Theoretical and applied genetics Theoretische und angewandte Genetik* 2004, **109**(8):1610-1619.
45. Tuyen DD, Lal SK, Xu DH: **Identification of a major QTL allele from wild soybean (Glycine soja Sieb. & Zucc.) for increasing alkaline salt tolerance in soybean**. *TAG Theoretical and applied genetics Theoretische und angewandte Genetik* 2010, **121**(2):229-236.
46. Muchero W, Ehlers JD, Roberts PA: **Restriction site polymorphism-based candidate gene mapping for seedling drought tolerance in cowpea [Vigna unguiculata (L.) Walp.]**. *TAG Theoretical and applied genetics Theoretische und angewandte Genetik* 2010, **120**(3):509-518.
47. Varshney RK, Close TJ, Singh NK, Hoisington DA, Cook DR: **Orphan legume crops enter the genomics era!** *Current opinion in plant biology* 2009, **12**(2):202-210.
48. Young ND, Debelle F, Oldroyd GED, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KFX, Gouzy J, Schoof H *et al*: **The Medicago genome provides insight into the evolution of rhizobial symbioses**. *Nature* 2011, **480**(7378):520-524.
49. Zhu H, Choi HK, Cook DR, Shoemaker RC: **Bridging model and crop legumes through comparative genomics**. *Plant physiology* 2005, **137**(4):1189-1196.
50. Young ND, Udvardi M: **Translating Medicago truncatula genomics to crop legumes**. *Current opinion in plant biology* 2009, **12**(2):193-201.
51. Cannon SB, May GD, Jackson SA: **Three sequenced legume genomes and many crop species: rich opportunities for translational genomics**. *Plant physiology* 2009, **151**(3):970-977.

Chapter 2

Identifying nitrate responsive NAC transcription factors in the transcriptome of *Medicago truncatula* root nodule using the Gene Expression Atlas

Authors: Vidyadhar Karmarkar[1,§], Nick Krom[2], Robin van Velzen[1], Erik Limpens[1], Ton Bisseling[1,3] and Rene Geurts[1]

Affiliations:

1. Laboratory of Molecular Biology, Wageningen University, Wageninengen, 6708PB , The Netherlands.

2. The Samuel Roberts Noble Foundation, Ardmore, OK, 73401, USA.

3. Department of Zoology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia

FOOTNOTE:

§ Current Address: Max Planck Institute for Plant Breeding Research, Carl-von-Linne-Weg 10, 50829 Cologne, Germany.

Abstract

The *Medicago truncatula* Gene Expression Atlas is a community resource that contains transcriptome data for various chemical, biological and physiological perturbed conditions. Particularly, it contains data on nitrate-induced changes in the transcriptome of *M. truncatula* nodules and roots. Hence, we performed data mining aiming to identify transcription factors differentially expressed upon nitrate treatment. Among the ~1,300 transcription factors represented on the microarray used to compile the Gene Expression Atlas, we identified members of NAC, MYB, AP2-EREBP, WRKY and bHLH families to be highly responsive to nitrate. Among these, two members of NAC (NAM, ATAF, CUC) family showed the highest upregulation within 48 hours after application of exogenous nitrate to the plants.

Introduction

The *Medicago truncatula* gene expression atlas (Gene Atlas) is an Affymetrix microarray based community resource, which contains expression data derived from 50,900 probes that represent the majority of genes in this species. It is a comprehensive compendium that provides a global overview of gene expression in all major organs of this species [1]. It includes the transcriptome response of *M. truncatula* to various physical, biological and chemical treatments. Importantly, it includes expression profiles of 1,478 probe IDs that correspond to putative transcription factors. Hence, it is a very good resource for identifying differentially expressed genes across various treatments or genotypes and thus design hypothesis driven experiments.

Application of exogenous soil nitrate triggers root nodule senescence and inhibits symbiotic nitrogen fixation [2]. Although these negative effects of nitrate on nodule longevity and symbiotic nitrogen fixation are known for a few decades, the underlying molecular mechanisms are not well understood. Because transcription factors are master regulators of many plant biological processes, these are good targets for genetic manipulation through plant breeding and biotechnology. However, which transcription factors are responsive to nitrate are unknown. To know the nitrate responsive genes in general and transcription factors in particular, the Gene Atlas serves as a valuable resource because it possesses the expression information of genes responding to 48-hour nitrate treatment in root and nodule tissues.

Transcription factor proteins are usually defined as transcriptional switches that are capable to activate and/or repress transcription upon binding to specific DNA motifs. This function often is dependent upon protein–protein interactions, and transcription factors are known to act in homo and/or heteromeric complexes. A significant proportion of protein-encoding genes in a plant genome is dedicated to the control of gene expression. For example, in *Arabidopsis thaliana* more than 2,500 genes encode a putative transcription factor, which represents ~5% of protein encoding genes [3]. In other plant species, these numbers can even been higher. For example, for *M. truncatula* ~3,600 putative transcription factors have been annotated, whereas for soybean (*Glycine max*) this number is 5,671, representing ~12% of the protein encoded genes [4]. Plant transcription factors can be divided in about 60 families, of which half are plant specific [3, 4]. Although no specific family is particular over-represented in plants, some families have considerably expanded in different plant lineages.

This suggests that they are involved in the regulation of clade-specific functions. In *A. thaliana*, *M. truncatula* and *G. max* some of the largest families of transcription factors are MYB, bHLH (basic helix-loop-helix), AP2/EREBP (APETALA2/ethylene responsive element binding protein), and NAC, each represent over 100 members [Table 1]. However, the vast majority of these genes have not been functionally characterized yet.

bHLH transcription factors are named according their highly conserved domain of about 60 amino acids with two functionally distinct regions. bHLH proteins represent an ancient family found in fungi, plants and animals, but not in prokaryotes [5]. In *A. thaliana*, the bHLH family consists of 147 members spanning 12 sub-families [6]. The bHLH members regulate diverse range of functions including phytochrome-mediated light signaling, anthocyanin biosynthesis, hormone signaling, abiotic stress, flowering time and development [listed in 7]. However, a recent review on bHLH in plant development and metabolism is lacking. In legumes only two bHLH-type transcription factors with a symbiotic function have characterized; *MtbHLH1* and *MtbHLH476* of *M. truncatula* [8-10]. The latter is a positive regulator of root nodule formation and primary responsive to a short-term (1 h) exogenous cytokinin treatment (10^{-7} M benzyl amino purine)[10]. However, its precise spatial and temporal expression pattern remains elusive, mainly due too low expression levels. In contrast, *MtbHLH1* is highly expressed in nodular vascular bundles. Also, the gene is active in those cells in the central zone of the nodule that are not infected by rhizobium [8]. Altering *MtbHLH1* expression affects vascular bundle formation, suggesting a key regulatory role of this transcription factor in this developmental process. Increased expression of *MtbHLH1* occurs at early stages of root nodule primordium formation, when *MtbHLH1* transcripts are specially accumulating in primordial cells that originate from pericycle cells [8, 9].

AP2-EREBP family, consisting of 147 members, representing ~9% of all transcription factors in *A. thaliana* [reviewed in 11]. AP2/EREBP proteins have been implicated in hormone, sugar and redox signaling involved in abiotic stress response. These proteins are characterized by the presence of AP2 DNA binding domain and is sub-divided into four subfamilies; AP2, DREB, ERF and RAV, respectively. Despite the size of this family, only a limited number of genes have been functionally characterized. In legume root nodule formation these include two genes – *MtERN1* and *MtEFD*. Both genes have been identified *M. truncatula* and are essential for root nodule formation [12-14] Furthermore, AP2/EREBP transcription factors of the DREB family have been identified in chickpea (*Cicer arietinum CaCAP2*) and in soybean (GmDREB2A;2) that upon ectopic expression in *A. thaliana* enhance growth an tolerance to salt [15-16]. Generally it is thought that such transcription

factors can be exploited in a biotech approach to increase salt tolerance [17].

MYB transcription factors possess a highly conserved DNA-binding domain [18]. The MYB proteins are classified based on the number of adjacent repeats of amino acids. Each sequence of these repeats consisting of ~52 amino acids is repeated 1-4 times. Based on the number of repeats, a suffix is added in the nomenclature of MYBs e.g. R1, R2, R3 and R4. In plants, the R2R3-MYB proteins are most common and possess a modular structure. The N-terminal region consists of the DNA-binding or the MYB domain whereas the C-terminal constitutes the activation or repression domain. Based on the conservation in the N- and C- terminal domains, the R2R3 MYBs have been classified into 25 subgroups and over 100 members. These members regulate diverse functions ranging from regulation of secondary metabolism, cell fate, cell identity, plant development and biotic/abiotic stress [reviewed in 18]. In *M. truncatula*, MYBs were shown to regulate proanthocyanidin biosynthesis [19-21] and were upregulated in response to salt stress [22]. However, no MYBs have been found to function in root nodule symbiosis.

WRKY transcription factors constitute one of the largest families in plants [23]. The characteristic feature of WRKY transcription factors is their “WRKY” DNA-binding domain based on the conservation of the tryptophan (W), arginine (R), lysine (K) and tyrosine (Y) amino acid motif in the N-terminus of the proteins. The WRKY domain is ~60 amino acids in length with a characteristic zinc-finger at the C-terminal. These proteins are divided into 5 sub-groups, which regulate diverse functions including biotic/abiotic stress, seed development, seed dormancy and germination, senescence and plant development [reviewed in 23]. In *M. truncatula*, WRKY genes were shown to regulate secondary cell wall formation and biomass, after-ripening and defense response in seeds and secondary metabolism [24-25].

NAC (NAM, ATAF, CUC) are plant specific transcription factors with a conserved N-terminal but variable C-terminal domains [26]. NAC genes are known to play a role in plant development, biotic and abiotic stress and senescence [27-28]. The role of NACs in senescence is particularly important because this family was significantly over-represented in three different transcriptomics studies on senescing leaves of *A. thaliana* [29-31]. In addition, functional characterization of several NAC members revealed their role in regulating leaf senescence in various species *A. thaliana*, *Oryza sativa*, *Gossypium herbaceum*, *Triticum aestivum*, *Brassica napus*, *Hordeum vulgare* and *Bambusa emiensis*

[32-37]. In *M. truncatula*, NACs were shown to be upregulated in response to salt stress [22] and regulate stress tolerance and nodule senescence [37].

Here, we mined the *M. truncatula* Gene Atlas for expression changes of transcription factor genes upon 48-hour treatment with ammonium nitrate. We present an overview of the data mining study with a particular emphasis on the nitrate response of members of AP2-EREBP, bHLH, NAC and MYB families.

Results and Discussion

Transcription factors on the M. truncatula GeneChip

The *M. truncatula* genome encodes ~ 3,600 putative transcription factors [38], which are divided over 57 families. On the Affimetrix GeneChip only 1,478 probe sets target a putative transcription factor gene. Aligning these probe sequences to the annotated *M. truncatula* genome (IMGAG Mtv3.5) revealed that 197 probes (~15%) are not unique, indicating that the GeneChip represents only 1,281 genes encoding transcription factors evenly distributed over the different gene families (table 1). Although this is only a fraction (~35%) of the total number of transcription factors encoded by the *M. truncatula* genome, the Medicago Gene Atlas is still a rich resource for gene mining in relation to rhizobium symbiosis, as the genes represented on the GeneChip are biased towards genes expressed in root and nodule tissues. This is because the GeneChip was originally designed based on ~200,000 EST sequences generated from roots and nodules tissues that were isolated from plants grown under diverse experimental conditions.

Mining the M. truncatula Gene Atlas to identify nitrate responsive transcription factors

A comprehensive gene expression atlas based on the *M. truncatula* GeneChip is publicly available and accessible via a webserver (http://mtgea.noble.org) [1]. We performed data mining of this Gene Atlas to identify nitrate responsive transcription factors. To this end, we used the following settings – the untreated nodule samples at 14 days post inoculation (dpi) and 16 dpi nodules treated for 48 hours with nitrate (of unknown concentration). We checked the expression profile of 1,478 probe IDs that correspond to transcription factors. On comparing the expression values of nitrate treated versus untreated nodules, we identified

Table1: Transcription factor families in *Arabidopsis thaliana* (arabidopsis), *Glycine max* (soybean), *Medicago truncatula* (medicago), and the number of genes that are represented on the *M. truncatula* gene chip.

Transcription factor family	arabidopsis	soybean	medicago	Medicago Gene Chip
MYB	303	791	320	155
C2H2	173	395	218	73
bHLH	172	393	194	93
AP2-EREB	146	381	246	90
NAC	114	208	119	63
HOMEODOMAIN / HOMEOBOX	112	319	173	37
MADS	109	212	136	44
C2C2	99	216	127	65
BTB/POZ	98	145	73	1
BZIP	78	176	87	69
WRKY	73	197	129	65
ABI3/VP1	71	78	107	24
C3H-type	69	147	88	38
CCHC	66	144	189	2
TPR	65	319	106	n.d.
PHD	55	222	133	24
AUX-IAA-ARF	51	129	78	42
AS2	43	92	60	7
CCAAT	38	106	30	34
GRAS	33	130	77	37
SNF2	33	69	43	4
BROMODOMAIN	29	57	46	n.d.
JUMONJI	21	77	40	7
ZF-HD	17	54	18	19
TCP	6	65	25	16
others	241	561	830	272
TOTAL	**2,315**	**5,683**	**3,692**	**1,281**

120 probe IDs representing 114 genes that displayed a 2-fold increase in expression, and 56 probe IDs representing 49 genes that 2-fold decreased in expression. Taken together, this analysis showed that the expression in root nodules of ~12% of the transcription factors, as presented on the *M. truncatula* GeneChip, is affected upon application of exogenous nitrate to the plant root.

To compare the nitrate-dependent upregulation of transcription factor genes between roots and nodules, we mined the *M. truncatula* gene expression atlas to compare the expression differences of the 1,478 probe IDs in untreated and nitrate treated roots with those of nodules. We identified 185 probe IDs that showed 2-fold change in root expression upon nitrate treatment [data not shown]. Next, we compared all the probe ids that show ≥ 2 fold

change in roots and nodules. In this list, we found 16 probe IDs that showed 2x fold change in roots and nodules. In Table 2, we present the top 25 candidates of up- and down-regulated genes from the nitrate treated nodules and roots [Table 2]. From this list of top 25 candidates, only 4 probe IDs, representing less than 10% of the transcription factors that are affected in root nodules upon nitrate treatment, are also affected in roots. This suggests that the majority of nitrate responsive transcription factor genes identified are expressed in the nodules or roots.

Interestingly, the 6 most affected genes of which the expression was down regulated belong to the bHLH family. Of only of one gene, Medtr3g111290.1, the expression change roots upon nitrate was above threshold (2-fold change) [Table 3]. This suggests that the remaining 5 genes are responsive in nodules and not in roots. Among the down-regulated genes in nodules, is *MtbHLH1* [8] of which its expression drops ~5-fold. *MtbHLH1* is essential for vascular development in the root nodule, and down regulation of this gene upon nitrate treatment may hint in abortion of nodule growth.

To get first insight in biological processes possibly controlled by these transcription factors, we determined whether *A. thaliana* has putative orthologs. To do so, a comparative approach using OrthoMCL was used to identify all transcription factors of *A. thaliana*, woodland strawberry (*Fragaria vesca*) and castor bean (*Ricinus communis*) that are homologous to the *M. truncatula* transcription factors annotated in Mt3.5 and present on the GeneChip. Woodland strawberry and castor bean were included in this study as they form bridging species in a phylogenetic context between *M. truncatula* and *A. thaliana*. This resulted in 622 putative orthology groups that contained at least one *M. truncatula* transcription factor gene. Next, we determined whether the 6 down regulated bHLH TFs have putative *A. thaliana* orthologs. Only for two bHLH genes, Medtr3g099620.1 and Medtr3g111290.1, putative orthologs in *A. thaliana* could be found, however with yet unknown functions [Table 3]. The *M. truncatula* gene of which the expression is most affected, Medtr2g104490.1, seems to be specific for legumes and/or *M. truncatula,* as no orthologs are present in any of the 3 non-legume species investigated. Also, this gene was not expressed in roots. Taken together, it may suggest that it has a symbiosis specific function.

Table 2: Expression profile in root and nodules of the top 25 transcription factors of which the expression is affected in root nodules upon nitrate treatment vs untreated nodules. In red: upregulated genes, in green: down regulated genes.

Transcription factor	Probeset	Gene.ID Mt3.5	Nod 14dpi	Nod 16dpi+NO3	Fold Change	Root	Root+NO3	Fold Change
NAC	Mtr.28491.1.S1_at	contig_76140_1.1	10,9	885,4	81,0	9,5	23,8	2,5
NAC	Mtr.32364.1.S1_at	contig_69267_1.1	12,4	931,9	75,0	20,6	28,5	1,4
WRKY	Mtr.48109.1.S1_at	Medtr3g106060.1	14,0	197,7	14,1	19,4	19,8	1,0
AP2-EREBP	Mtr.42129.1.S1_at	NA	17,7	203,6	11,5	80,9	14,3	0,2
NAC	Mtr.19019.1.S1_at	Medtr4g081870.1	336,7	2438,8	7,2	124,3	166,6	1,3
WRKY	Mtr.44416.1.S1_at	NA	24,7	168,8	6,8	27,3	18,2	0,7
WRKY	Mtr.42336.1.S1_at	Medtr2g033820.1	44,8	251,2	5,6	51,0	15,9	0,3
HD / ZIP	Mtr.1597.1.S1_at	Medtr4g126900.1	8,5	42,4	5,0	10,8	10,5	1,0
AP2/EREBP	Mtr.11570.1.S1_at	Medtr1g090170.1	40,0	196,5	4,9	685,7	2003,7	2,9
Putative Novel TF	Mtr.50075.1.S1_s_at	Medtr4g017030.1	162,1	784,6	4,8	2007,9	975,2	0,5
AP2/EREBP	Mtr.37973.1.S1_at	Medtr1g098460.1	398,4	1928,0	4,8	252,1	249,1	1,0
C2H2 (ZF)	Mtr.7762.1.S1_at	NA	13,1	63,3	4,8	14,9	14,8	1,0
MYB / HD-like	Mtr.15018.1.S1_at	Medtr1g086530.1	19,3	91,9	4,8	12,0	8,1	0,7
AS2	Mtr.27551.1.S1_at	Medtr5g015880.1	11,2	53,0	4,7	18,7	16,0	0,9
HSF	Mtr.35193.1.S1_s_at	contig_81335_1.1	17,2	79,2	4,6	25,8	24,8	1,0
WRKY	Mtr.32312.1.S1_at	Medtr3g104750.1	27,6	127,1	4,6	43,2	75,9	1,8
NAC	Mtr.38349.1.S1_at	Medtr5g090970.1	21,0	93,9	4,5	20,7	45,6	2,2
HD / ZIP	Mtr.18769.1.S1_at	Medtr8g026960.1	245,3	1062,6	4,3	207,0	70,2	0,3
C2H2 (ZF)	Mtr.51614.1.S1_at	Medtr2g093960.1	1268,0	5245,0	4,1	66,7	19,5	0,3
GRAS	Mtr.50733.1.S1_at	Medtr2g034250.1	32,6	131,6	4,0	55,8	67,8	1,2
MYB / HD-like	Mtr.5277.1.S1_at	NA	10,2	41,0	4,0	14,5	11,6	0,8
MYB	Mtr.38757.1.S1_at	Medtr2g033170.1	93,2	372,2	4,0	35,4	19,6	0,6
NAC	Mtr.44141.1.S1_at	NA	13,6	52,6	3,9	9,0	10,5	1,2
MYB / HD-like	Mtr.15010.1.S1_s_at	Medtr1g086510.1	140,8	534,0	3,8	123,0	99,3	0,8
MYB	Mtr.38757.1.S1_at	Medtr2g033170.1	93,2	372,2	4,0	35,4	19,6	0,6
bHLH	Mtr.253.1.S1_at	Medtr5g014520.1	164,8	61,5	0,4	68,5	15,4	0,2
MYB / HD-like	Mtr.15339.1.S1_at	Medtr1g043050.1	17,2	6,4	0,4	5,8	5,6	1,0
AP2/EREBP	Mtr.35480.1.S1_at	Medtr7g085810.1	253,8	92,3	0,4	28,9	46,6	1,6
bZIP	Mtr.42559.1.S1_at	Medtr5g060940.1	58,2	20,9	0,4	71,3	51,2	0,7
C2H2 (ZF)	Mtr.32254.1.S1_at	Medtr7g082260.1	34,0	11,7	0,3	14,2	14,1	1,0
C2C2 (ZF) / DOF	Mtr.45038.1.S1_at	contig_81843_1.1	547,4	184,3	0,3	182,7	187,9	1,0
C3H (ZF)	Mtr.7146.1.S1_at	Medtr3g070780.1	204,5	67,2	0,3	80,1	64,3	0,8
bHLH	Mtr.1885.1.S1_at	Medtr4g098250.1	495,6	161,1	0,3	308,5	311,3	1,0
MYB	Mtr.26044.1.S1_s_at	Medtr7g118330.1	67,1	21,6	0,3	479,9	116,7	0,2
bHLH	Mtr.14710.1.S1_at	Medtr5g030770.1	195,9	60,6	0,3	76,7	60,8	0,8
WRKY	Mtr.27549.1.S1_at	Medtr7g009730.1	236,5	69,2	0,3	73,5	88,4	1,2
HD	Mtr.29074.1.S1_at	Medtr1g016490.1	34,7	9,4	0,3	9,3	9,1	1,0
C2H2 (ZF)	Mtr.50786.1.S1_at	Medtr4g053170.1	471,5	127,8	0,3	301,1	191,0	0,6
PHD	Mtr.30792.1.S1_at	Medtr3g117270.1	89,4	23,9	0,3	9,5	9,0	0,9
GRAS	Mtr.13410.1.S1_at	Medtr4g076140.1	81,4	21,2	0,3	26,1	51,1	2,0
AP2-EREBP	Mtr.5385.1.S1_at	contig_67698_2.1	48,0	12,5	0,3	96,0	56,4	0,6
HD	Mtr.6737.1.S1_s_at	Medtr3g106400.1	46,9	11,9	0,3	24,8	31,4	1,3
C2H2 (ZF)	Mtr.38404.1.S1_at	NA	1085,3	263,7	0,2	7,0	8,1	1,2
bHLH	Mtr.14760.1.S1_at	Medtr4g092700.1	433,6	100,8	0,2	35,8	34,3	1,0
bHLH	Mtr.27133.1.S1_at	contig_75497_1.1	126,8	26,4	0,2	35,0	18,5	0,5
bHLH	Mtr.44005.1.S1_at	Medtr3g111290.1	235,3	48,9	0,2	308,0	102,6	0,3
bHLH	Mtr.10993.1.S1_at	Medtr3g099620.1	2598,9	397,9	0,2	897,4	1279,3	1,4
MYB	Mtr.40836.1.S1_at	Medtr7g061550.1	117,6	17,9	0,2	20,5	41,7	2,0
bHLH	Mtr.23988.1.S1_at	Medtr1g019240.1	544,1	63,9	0,1	14,3	20,6	1,4
bHLH	Mtr.3879.1.S1_at	Medtr2g104490.1	816,6	44,9	0,1	8,7	11,4	1,3

Table 3: bHLH transcription factors that are highly expressed in root nodules, and down regulated upon application of exogenous nitrate nitrate.

Transcription factor	Probeset	Gene.ID Mt3.5 /Mt4.0	Nod 14 dpi	Nod 16dpi+NO3	FC	Root	Root +NO3	FC	Arabidopsis Ortho
bHLH	Mtr.3879.1.S1_at	Medtr2g104490.1	816,6	44,9	**-18,2**	8,7	11,4	**-0,8**	no ortholog
bHLH	Mtr.23988.1.S1_at	Medtr1g019240.1	544,1	63,9	**-8,5**	14,3	20,6	**-0,7**	no ortholog
MtbHLH1	Mtr.10993.1.S1_at	Medtr3g099620.1	2598,9	397,9	**-6,5**	897,4	1279,3	**-0,7**	AT1G22490.1, AT1G72210.1
bHLH	Mtr.44005.1.S1_at	Medtr3g111290.1	235,3	48,9	**-4,8**	308,0	102,6	**-3,0**	AT5G10570.1, AT5G65640.1
bHLH	Mtr.27133.1.S1_at	contig_75497_1.1 / Medtr8g024790.1	126,8	26,4	**-4,8**	35,0	18,5	**-1,9**	n.d.
bHLH	Mtr.14760.1.S1_at	Medtr4g092700.1	433,6	100,8	**-4,3**	35,8	34,3	**-1,0**	no ortholog

Members of the NAC, WRKY, bHLH, and AP2-EREBP families are highly upregulated in nitrate treated root nodules

In the NAC family, we identified 13 genes that showed fold change > 2x. Among these, the highest induced transcription factors had the probe ids Mtr.28491.1.S1_at and Mtr.32364.1.S1_at. Based on the OrthoMCL pipeline described above, we could not identify any *A. thaliana* orthologs for the genes represented by these probe IDs. Interestingly though, we identified two NAC genes with probe ids Mtr.19019.1.S1_at and Mtr.38349.1.S1_at that showed 7.2x and 4.5x fold upregulation respectively. Based on OrthoMCL, the *A. thaliana* orthologs of these genes were AT1G69490 (*AtNAP*) and AT2G43000 (*JUB1*) respectively. *AtNAP* was shown or regulate leaf and fruit senescence in *A. thaliana* [39-40]. Another member of the NAP family, *BeNAC1*, from bamboo (*Bambusa emeiensis*) was shown to be important for leaf senescence [36]. Constitutive overexpression of *BeNAC1* in *A. thaliana* resulted in premature senescence [36]. Similarly, *JUB1* was shown to be important to regulate senescence in *A. thaliana* [41-42]. *JUB1* was also shown to be responsive to reactive oxygen species, which are important signaling intermediates in plant senescence [43].

Table 4: NAC transcription factors that are induced in root nodules upon application of exogenous nitrate.

Transcription factor	Probeset	Gene.ID Mt3.5v4 / Mt4.0	Nod 14dpi	Nod 16dpi+NO3	FC	Root	Root+NO3	FC	ARABIDOPSIS ortho
NAC	Mtr.28491.1.S1_at	contig_76140_1.1 / Medtr1g069805.1	10,9	885,4	81,0	9,5	23,8	2,5	n.d.
NAC	Mtr.32364.1.S1_at	contig_69267_1.1 / Medtr7g097090.1	12,4	931,9	75,0	20,6	28,5	1,4	n.d.
NAC	Mtr.19019.1.S1_at	Medtr4g081870.1	336,7	2438,8	7,2	124,3	166,6	1,3	AT1G69490.1 **AtNAP**
NAC	Mtr.38349.1.S1_at	Medtr5g090970.1	21,0	93,9	4,5	20,7	45,6	2,2	AT2G43000.1 **AtJUB1**
NAC	Mtr.44141.1.S1_at	n.a. / Medtr4g094302.1	13,6	52,6	3,9	9,0	10,5	1,2	n.d.
NAC	Mtr.33363.1.S1_at	n.a. / Medtr3g435150.1	19,2	64,6	3,4	40,3	13,9	0,3	n.d.
NAC	Mtr.31800.1.S1_at	Medtr1g008740.1	28,3	80,9	2,9	14,2	8,7	0,6	AT2G24430.1
NAC	Mtr.17710.1.S1_at	Medtr8g069160.1	19,1	53,9	2,8	110,7	112,1	1,0	AT1G28470.1 / AT4G28500.1
NAC	Mtr.41968.1.S1_at	n.a. /Medtr6g011860.1	154,8	393,4	2,5	6,8	6,3	0,9	n.d.
NAC	Mtr.43258.1.S1_s_at	contig_65520_1.1 / Medtr2g079990.2	115,1	262,2	2,3	80,1	228,9	2,9	n.d.
NAC	Mtr.43257.1.S1_at	n.a. / Medtr2g079990.1	486,9	1080,3	2,2	136,3	571,1	4,2	n.d.
NAC	Mtr.42785.1.S1_at	Medtr4g075980.1	69,0	143,3	2,1	31,3	78,3	2,5	AT2G17040.1
NAC	Mtr.8698.1.S1_at	contig_239170_1.1 / Medtr1g097300.1	574,4	1189,5	2,1	1111,7	784,2	0,7	n.d.

In total 16 WRKY-type transcription factors have been identified that showed > 2x fold upregulation upon nitrate treatment (Table 5). Among this list, we could not find any *A. thaliana* ortholog that may be involved in abiotic stress pathways. In contrast, all the *A. thaliana* orthologs of most of the candidates were involved in biotic stress. This is not surprising because WRKY genes are one of the best characterized for their response to pathogen challenge [23]. For instance, Medtr2g033820 (represented by the probe ID Mtr.42336.1.S1_at) that showed a fold change of 5.6x is orthologous to *A. thaliana* ortholog *WRKY48*. The knockout mutants lines of this gene, *wrky48*, displayed a higher resistance to the bacterial *Psuedomonas syringae* [44]. Similarly another WRKY gene with the probe id Mtr.32312.1.S1_at showed 4.6x fold increase upon nitrate application. The closest homolog in *A. thaliana* of this gene was identified to be *WRKY51*. *WRKY51* was also shown to be important for low-oleic acid mediated repression of JA-induced defense responses [45].

Table 5: WRKY transcription factors that are induced in root nodules upon application of exogenous nitrate.

Transcription factor	Probeset	Gene.ID Mt3.5 / Mt4.0	Nod 14dpi	Nod 16dpi+NO3	FC	Root	root+NO3	FC	ARABIDOPSIS ortho
WRKY	Mtr.48109.1.S1_at	Medtr3g106060.1	14,0	197,7	14,1	19,4	19,8	1,0	no ortholog
WRKY	Mtr.44416.1.S1_at	n.a. / Medtr7g028415.1	24,7	168,8	6,8	27,3	18,2	0,7	n.d.
WRKY	Mtr.42336.1.S1_at	Medtr2g033820.1	44,8	251,2	5,6	51,0	15,9	0,3	AT5G49520.1 **WRKY48**
WRKY	Mtr.32312.1.S1_at	Medtr3g104750.1	27,6	127,1	4,6	43,2	75,9	1,8	AT5G26170.1
WRKY	Mtr.45888.1.S1_s_at	Medtr3g095040.1	34,6	104,5	3,0	36,3	98,9	**2,7**	no ortholog
WRKY	Mtr.43171.1.S1_at	Medtr4g007060.1	153,8	448,5	2,9	82,8	141,1	1,7	AT1G80840.1 **WRKY40**
WRKY	Mtr.12610.1.S1_at	contig_168934_1.1 / Medtr3g093830.1	704,9	1958,2	2,8	579,8	1919,9	**3,3**	n.d.
WRKY	Mtr.12.1.S1_at	Medtr5g074400.1	577,3	1579,3	2,7	411,4	380,0	0,9	AT2G30250.1 **WRKY25**
WRKY	Mtr.32279.1.S1_at	Medtr3g031220.1	91,9	235,5	2,6	216,7	84,1	0,4	no ortholog
WRKY	Mtr.28804.1.S1_at	Medtr4g132430.1	64,5	159,4	2,5	125,4	119,1	0,9	AT4G04450.1
WRKY	Mtr.40890.1.S1_at	contig_75277_2.1 / Medtr2g105060.1	606,7	1444,7	2,4	127,8	44,7	0,3	n.d.
WRKY	Mtr.48688.1.S1_at	Medtr2g013950.1	162,7	360,9	2,2	135,0	231,0	1,7	AT1G28280.1
WRKY	Mtr.40917.1.S1_at	Medtr7g071120.1	171,5	377,0	2,2	271,2	212,3	0,8	AT2G38470.1 **WRKY33**
WRKY	Mtr.13044.1.S1_at	Medtr2g045360.1	126,4	277,0	2,2	239,6	354,5	1,5	no ortholog
WRKY	Mtr.37425.1.S1_at	n.a. / Medtr2g023930.1	1023,3	2170,2	2,1	1373,5	1556,0	1,1	n.d.
WRKY	Mtr.10976.1.S1_at	n.a. / Medtr4g094908.1	1368,9	2756,6	2,0	1720,8	2855,7	1,7	n.d.

At least 16 AP2-EREB transcription factors have been identified with a 2-fold expression change in root nodules upon nitrate treatment. Among these nitrate-responsive AP2-EREBPs, the function of *A. thaliana* orthologs was unknown, with the exception of Medtr3g095190 (probe id Mtr.39021.1.S1_at). The *A. thaliana* ortholog of this gene is *ABR1*, which is a repressor of ABA responses [46]. Interestingly, we identified two probe IDs, Mtr.42129.1.S1_at (Medtr1g069805.1) and Mtr.37973.1.S1_at (Medtr7g097090.1), which were identified to represent a *Lotus japonicus* transcription factor, *LjERF17*. This gene has shown to be expressed in nodules and induced in roots upon JA signaling [47]. Based on the *LjERF17* phenotype and the nitrate responsiveness of Medtr6g037610.1 (Mtr.42129.1.S1_at) and Medtr1g098460.1 (Mtr.37973.1.S1_at), it is tempting to speculate that knockdown of these AP2-EREBPs could result in nitrate tolerant nodulation.

Table 6: AP2-EREBP transcription factors that are induced in root nodules upon application of exogenous nitrate.

Transcription factor	Probeset	Gene.ID Mt3.5 / Mt4.0	Nod 14dpi	Nod 16dpi+NO3	FC	root	root+NO3	FC	ARABIDOPSIS ortho
AP2-EREBP	Mtr.42129.1.S1_at	n.a. / Medtr6g037610.1	17,7	203,6	11,5	80,9	14,3	0,2	n.d.
AP2-EREBP	Mtr.11570.1.S1_at	Medtr1g090170.1	40,0	196,5	4,9	685,7	2003,7	**2,9**	AT4G13620.1
AP2/EREBP	Mtr.37973.1.S1_at	Medtr1g098460.1	398,4	1928,0	4,8	252,1	249,1	1,0	no ortholog
AP2/EREBP	Mtr.45730.1.S1_at	Medtr1g019110.1	197,9	605,1	3,1	270,6	601,9	**2,2**	AT1G46768.1
AP2/EREBP	Mtr.45232.1.S1_at	Medtr7g070220.1	18,1	53,3	2,9	19,6	17,5	0,9	no ortholog
AP2/EREBP	Mtr.16212.1.S1_at	Medtr8g092460.1	17,3	49,3	2,9	8,7	9,0	1,0	no ortholog
AP2/EREBP	Mtr.36431.1.S1_at	contig_113760_1.1 / Medtr6g033255.1	52,2	136,1	2,6	77,3	36,9	0,5	n.d.
AP2/EREBP	Mtr.5750.1.S1_at	contig_52379_1.1 / Medtr7g096810.1	21,1	53,6	2,5	20,6	8,1	0,4	n.d.
AP2/EREBP	Mtr.51291.1.S1_at	Medtr3g102100.1	411,3	1042,1	2,5	310,4	313,5	1,0	AT1G46768.1
AP2/EREBP	Mtr.11163.1.S1_at	Medtr2g093060.1	146,5	351,7	2,4	661,3	1267,9	1,9	AT2G28550.3
AP2/EREBP	Mtr.13273.1.S1_at	Medtr3g072610.1	90,0	207,8	2,3	83,1	19,5	0,2	AT1G44830.1
AP2/EREBP	Mtr.31334.1.S1_at	contig_9736_1.1 / Medtr1g028560.1	12,6	28,3	2,3	95,7	157,3	1,6	n.d.
AP2/EREBP	Mtr.39082.1.S1_at	Medtr5g016810.1	154,0	332,9	2,2	228,5	140,3	0,6	AT4G36920.1
AP2/EREBP	Mtr.39021.1.S1_at	Medtr3g095190.1	108,2	219,9	2,0	31,5	28,0	0,9	AT5G64750.1 **ABR1**
AP2/EREBP	Mtr.28159.1.S1_at	contig_76531_1.1 / Medtr1g101550.1	145,8	295,1	2,0	492,3	139,0	0,3	n.d.
AP2/EREBP	Mtr.12775.1.S1_at	Medtr1g101550.1	35,2	70,9	2,0	67,3	67,0	1,0	AT5G52020.1

Conclusion

Among the ~1,200 *M. truncatula* transcription factors, the members of the NAC, WRKY, bHLH and AP2-EREBP family were highly sensitive to nitrate treatment. Among these gene families, the NAC transcription factors Medtr1g069805.1 (Mtr.28491.1.S1_at) and Medtr7g097090.1 (Mtr.32364.1.S1_at) showed the highest fold upregulation upon nitrate treatment. Furthermore, both genes are basically nodule specific. Additionally, the bHLH family was unique amongst the other families because the members of this family showed maximum fold decrease upon nitrate treatment.

Material and Methods

Data retrieval and differential expression analysis

The data used for differential expression analysis was derived from untreated nodules at 14 dpi, 16 dpi nitrate-treated nodules, untreated roots and nitrate-treated roots. The absolute values for each probe ID was used for comparing the relative expression changes between treatments of interest. The fold change in expression for each probe id was calculated by the dividing the treatment sample by reference sample.

OrthoMCL analysis

To assess orthology, we downloaded all protein sequences from *M. truncatula*, *Fragaria vesca*, *Ricinus communis*, and *Arabidopsis thaliana* from Phytozome version 9.1 (www.phytozome.net) and analyzed these using the orthoMCL pipeline version 2.0.9 [48], based on blastn alignment and clustering with MCL (micans.org/mcl/) [49-50] with default settings.

References

1. Benedito VA, Torres-Jerez I, Murray JD, Andriankaja A, Allen S, Kakar K, Wandrey M, Verdier J, Zuber H, Ott T *et al*: **A gene expression atlas of the model legume Medicago truncatula**. *The Plant journal : for cell and molecular biology* 2008, **55**(3):504-513.
2. Chen PC, Phillips DA: **Induction of Root Nodule Senescence by Combined Nitrogen in Pisum sativum L**. *Plant physiology* 1977, **59**(3):440-442.
3. Riechmann JL, Ratcliffe OJ: **A genomic perspective on plant transcription factors**. *Current opinion in plant biology* 2000, **3**(5):423-434.
4. Young ND, Debelle F, Oldroyd GED, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KFX, Gouzy J, Schoof H *et al*: **The Medicago genome provides insight into the evolution of rhizobial symbioses**. *Nature* 2011, **480**(7378):520-524.
5. Stevens JD, Roalson EH, Skinner MK: **Phylogenetic and expression analysis of the basic helix-loop-helix transcription factor gene family: genomic approach to cellular differentiation**. *Differentiation; research in biological diversity* 2008, **76**(9):1006-1022.
6. Toledo-Ortiz G, Huq E, Quail PH: **The Arabidopsis basic/helix-loop-helix transcription factor family**. *The Plant cell* 2003, **15**(8):1749-1770.
7. Zhao H, Li X, Ma L: **Basic helix-loop-helix transcription factors and epidermal cell fate determination in Arabidopsis**. *Plant Signal Behav* 2012, **7**(12):1556-1560.
8. Godiard L, Lepage A, Moreau S, Laporte D, Verdenaud M, Timmers T, Gamas P: **MtbHLH1, a bHLH transcription factor involved in Medicago truncatula nodule vascular patterning and nodule to plant metabolic exchanges**. *The New phytologist* 2011, **191**(2):391-404.
9. Godiard L, Niebel A, Micheli F, Gouzy J, Ott T, Gamas P: **Identification of new potential regulators of the Medicago truncatula-Sinorhizobium meliloti symbiosis using a large-scale suppression subtractive hybridization approach**. *Molecular plant-microbe interactions : MPMI* 2007, **20**(3):321-332.
10. Ariel F, Brault-Hernandez M, Laffont C, Huault E, Brault M, Plet J, Moison M, Blanchet S, Ichante JL, Chabaud M *et al*: **Two direct targets of cytokinin signaling regulate symbiotic nodulation in Medicago truncatula**. *The Plant cell* 2012, **24**(9):3838-3852.
11. Dietz KJ, Vogel MO, Viehhauser A: **AP2/EREBP transcription factors are part of gene regulatory networks and integrate metabolic, hormonal and environmental signals in stress acclimation and retrograde signalling**. *Protoplasma* 2010, **245**(1-4):3-14.
12. Andriankaja A, Boisson-Dernier A, Frances L, Sauviac L, Jauneau A, Barker DG, de Carvalho-Niebel F: **AP2-ERF transcription factors mediate Nod factor dependent Mt ENOD11 activation in root hairs via a novel cis-regulatory motif**. *The Plant cell* 2007, **19**(9):2866-2885.
13. Vernie T, Moreau S, de Billy F, Plet J, Combier JP, Rogers C, Oldroyd G, Frugier F, Niebel A, Gamas P: **EFD Is an ERF transcription factor involved in the control of nodule number and differentiation in Medicago truncatula**. *The Plant cell* 2008, **20**(10):2696-2713.
14. Cerri MR, Frances L, Laloum T, Auriac MC, Niebel A, Oldroyd GE, Barker DG, Fournier J, de Carvalho-Niebel F: **Medicago truncatula ERN transcription factors: regulatory interplay with NSP1/NSP2 GRAS factors and expression dynamics throughout rhizobial infection**. *Plant physiology* 2012, **160**(4):2155-2172.
15. Mizoi J, Ohori T, Moriwaki T, Kidokoro S, Todaka D, Maruyama K, Kusakabe K, Osakabe Y, Shinozaki K, Yamaguchi-Shinozaki K: **GmDREB2A;2, a canonical**

DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN2-type transcription factor in soybean, is posttranslationally regulated and mediates dehydration-responsive element-dependent gene expression. *Plant physiology* 2013, **161**(1):346-361.

16. Shukla RK, Raha S, Tripathi V, Chattopadhyay D: **Expression of CAP2, an APETALA2-family transcription factor from chickpea, enhances growth and tolerance to dehydration and salt stress in transgenic tobacco**. *Plant physiology* 2006, **142**(1):113-123.
17. Hussain SS, Kayani MA, Amjad M: **Transcription factors as tools to engineer enhanced drought stress tolerance in plants**. *Biotechnology progress* 2011, **27**(2):297-306.
18. Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L: **MYB transcription factors in Arabidopsis**. *Trends Plant Sci* 2010, **15**(10):573-581.
19. Xie DY, Sharma SB, Dixon RA: **Anthocyanidin reductases from Medicago truncatula and Arabidopsis thaliana**. *Archives of biochemistry and biophysics* 2004, **422**(1):91-102.
20. Verdier J, Zhao J, Torres-Jerez I, Ge S, Liu C, He X, Mysore KS, Dixon RA, Udvardi MK: **MtPAR MYB transcription factor acts as an on switch for proanthocyanidin biosynthesis in Medicago truncatula**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(5):1766-1771.
21. Peel GJ, Pang Y, Modolo LV, Dixon RA: **The LAP1 MYB transcription factor orchestrates anthocyanidin biosynthesis and glycosylation in Medicago**. *The Plant journal : for cell and molecular biology* 2009, **59**(1):136-149.
22. Gruber V, Blanchet S, Diet A, Zahaf O, Boualem A, Kakar K, Alunni B, Udvardi M, Frugier F, Crespi M: **Identification of transcription factors involved in root apex responses to salt stress in Medicago truncatula**. *Molecular genetics and genomics : MGG* 2009, **281**(1):55-66.
23. Rushton PJ, Somssich IE, Ringler P, Shen QJ: **WRKY transcription factors**. *Trends Plant Sci* 2010, **15**(5):247-258.
24. Wang H, Avci U, Nakashima J, Hahn MG, Chen F, Dixon RA: **Mutation of WRKY transcription factors initiates pith secondary wall formation and increases stem biomass in dicotyledonous plants**. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(51):22338-22343.
25. Bolingue W, Rosnoblet C, Leprince O, Vu BL, Aubry C, Buitink J: **The MtSNF4b subunit of the sucrose non-fermenting-related kinase complex connects after-ripening and constitutive defense responses in seeds of Medicago truncatula**. *The Plant journal : for cell and molecular biology* 2010, **61**(5):792-803.
26. Olsen AN, Ernst HA, Leggio LL, Skriver K: **NAC transcription factors: structurally distinct, functionally diverse**. *Trends Plant Sci* 2005, **10**(2):79-87.
27. Nakashima K, Takasaki H, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K: **NAC transcription factors in plant abiotic stress responses**. *Biochimica et biophysica acta* 2012, **1819**(2):97-103.
28. Balazadeh S, Riano-Pachon DM, Mueller-Roeber B: **Transcription factors regulating leaf senescence in Arabidopsis thaliana**. *Plant Biol (Stuttg)* 2008, **10 Suppl 1**:63-75.
29. Guo Y, Cai Z, Gan S: **Transcriptome of Arabidopsis leaf senescence**. *Plant, cell & environment* 2004, **27**(5):521-549.
30. Breeze E, Harrison E, McHattie S, Hughes L, Hickman R, Hill C, Kiddle S, Kim YS, Penfold CA, Jenkins D *et al*: **High-resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals a distinct chronology of processes and regulation**. *The Plant cell* 2011, **23**(3):873-894.

31. Shah ST, Pang C, Fan S, Song M, Arain S, Yu S: **Isolation and expression profiling of GhNAC transcription factor genes in cotton (Gossypium hirsutum L.) during leaf senescence and in response to stresses**. *Gene* 2013, **531**(2):220-234.
32. Zhou Y, Huang W, Liu L, Chen T, Zhou F, Lin Y: **Identification and functional characterization of a rice NAC gene involved in the regulation of leaf senescence**. *BMC plant biology* 2013, **13**:132.
33. Ricachenevsky FK, Menguer PK, Sperotto RA: **kNACking on heaven's door: how important are NAC transcription factors for leaf senescence and Fe/Zn remobilization to seeds?** *Frontiers in plant science* 2013, **4**:226.
34. Zhong H, Guo QQ, Chen L, Ren F, Wang QQ, Zheng Y, Li XB: **Two Brassica napus genes encoding NAC transcription factors are involved in response to high-salinity stress**. *Plant cell reports* 2012, **31**(11):1991-2003.
35. Christiansen MW, Holm PB, Gregersen PL: **Characterization of barley (Hordeum vulgare L.) NAC transcription factors suggests conserved functions compared to both monocots and dicots**. *BMC research notes* 2011, **4**:302.
36. Chen Y, Qiu K, Kuai B, Ding Y: **Identification of an NAP-like transcription factor BeNAC1 regulating leaf senescence in bamboo (Bambusa emeiensis'Viridiflavus')**. *Physiologia plantarum* 2011, **142**(4):361-371.
37. de Zélicourt A, Diet A, Marion J, Laffont C, Ariel F, Moison M, Zahaf O, Crespi M, Gruber V, Frugier F: **Dual involvement of a Medicago truncatula NAC transcription factor in root abiotic stress response and symbiotic nodule senescence**. *The Plant Journal* 2012, **70**(2):220-230.
38. Libault M, Joshi T, Benedito VA, Xu D, Udvardi MK, Stacey G: **Legume transcription factor genes: what makes legumes so special?** *Plant physiology* 2009, **151**(3):991-1001.
39. Kou X, Watkins CB, Gan SS: **Arabidopsis AtNAP regulates fruit senescence**. *Journal of experimental botany* 2012, **63**(17):6139-6147.
40. Guo Y, Gan S: **AtNAP, a NAC family transcription factor, has an important role in leaf senescence**. *The Plant journal : for cell and molecular biology* 2006, **46**(4):601-612.
41. Wu A, Allu AD, Garapati P, Siddiqui H, Dortay H, Zanor MI, Asensi-Fabado MA, Munne-Bosch S, Antonio C, Tohge T *et al*: **JUNGBRUNNEN1, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in Arabidopsis**. *The Plant cell* 2012, **24**(2):482-506.
42. Shahnejat-Bushehri S, Mueller-Roeber B, Balazadeh S: **Arabidopsis NAC transcription factor JUNGBRUNNEN1 affects thermomemory-associated genes and enhances heat stress tolerance in primed and unprimed conditions**. *Plant Signal Behav* 2012, **7**(12):1518-1521.
43. Khanna-Chopra R: **Leaf senescence and abiotic stresses share reactive oxygen species-mediated chloroplast degradation**. *Protoplasma* 2012, **249**(3):469-481.
44. Xing DH, Lai ZB, Zheng ZY, Vinod KM, Fan BF, Chen ZX: **Stress- and pathogen-induced Arabidopsis WRKY48 is a transcriptional activator that represses plant basal defense**. *Molecular plant* 2008, **1**(3):459-470.
45. Gao QM, Venugopal S, Navarre D, Kachroo A: **Low oleic acid-derived repression of jasmonic acid-inducible defense responses requires the WRKY50 and WRKY51 proteins**. *Plant physiology* 2011, **155**(1):464-476.
46. Pandey GK, Grant JJ, Cheong YH, Kim BG, Li L, Luan S: **ABR1, an APETALA2-domain transcription factor that functions as a repressor of ABA response in Arabidopsis**. *Plant physiology* 2005, **139**(3):1185-1193.
47. Asamizu E, Shimoda Y, Kouchi H, Tabata S, Sato S: **A positive regulatory role for LjERF1 in the nodulation process is revealed by systematic analysis of nodule-**

associated transcription factors of Lotus japonicus. *Plant physiology* 2008, **147**(4):2030-2040.
48. Li L, Stoeckert CJ, Jr., Roos DS: **OrthoMCL: identification of ortholog groups for eukaryotic genomes**. *Genome research* 2003, **13**(9):2178-2189.
49. van Dongen S, Abreu-Goodger C: **Using MCL to extract clusters from networks**. *Methods Mol Biol* 2012, **804**:281-295.
50. Enright AJ, Van Dongen S, Ouzounis CA: **An efficient algorithm for large-scale detection of protein families**. *Nucleic acids research* 2002, **30**(7):1575-1584.

Chapter 3

Nitrate inhibits root nodule development by recruiting EIN2/SKL-MtNAC074-VAMP721 regulatory chain

Authors: Vidyadhar Karmarkar[1,§], Margo Smit[1], Cezary Smaczniak[1,†], Kerstin Kaufmann[1,†,‡], Marco Busscher[1], Erik Limpens[1], Ton Bisseling[1,3] René Geurts[1]

Affiliations:

1. Laboratory of Molecular Biology, Department of Plant Science, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands,.
2. The Samuel Roberts Noble Foundation, Ardmore, OK, 73401, USA.
3. College of Science, King Saud University, Post Office Box 2455, Riyadh 11451, Saudi Arabia.

FOOTNOTE:

§ Current Address: Max Planck Institute for Plant Breeding Research, Carl-von-Linne-Weg 10, 50829 Cologne, Germany.

‡ Current Address: Institute for Biochemistry and Biology, University of Potsdam, Potsdam-Golm, 14476, Germany.

† These authors equally contributed to the work

This chapter has been submitted for publication.

Abstract

Endocytosis of the rhizobial bacterium invading legume root nodule results in a plant derived membrane compartment called the symbiosome. Normal symbisome development is adversely affected by exogenous soil nitrate. However, the molecular insights to the transcriptional regulation of nitrate-induced arrest in symbiosome development are lacking. In the present study, using the model legume *Medicago truncatula*, we have identified a member of NAC (NAM, ATAF, CUC) family, *MtNAC074*, which is highly upregulated upon nitrate treatment. The nitrate-dependent accumulation of *MtNAC074* transcripts requires ethylene biosynthesis and signaling. We further show that MtNAC074 directly binds to the promoters of *MtVAMPs.* Taken together, we propose a nitrate-ethylene-MtNAC074 regulatory chain, which is recruited upon nitrate application leading to symbiosome arrest.

Introduction

Legumes are critical for the global nitrogen economy because of their ability to establish symbiosis with nitrogen fixing bacteria called rhizobia. Symbiotic nitrogen fixation is an energetically expensive process. The expense is sustained only when the benefit-to-cost trade-off is in favor of the host legume. Hence, legumes have evolved a tight genetic regulatory mechanism to control root nodule number by sensing external and internal cues.

The number of nodules formed on a root system is controlled systemically through root-shoot communication [1]. This process is called autoregulation of nodulation. A major external cue that regulates nodule number is soil nitrate, which has a dual affect on symbiotic nitrogen fixation. While nitrogen-limiting conditions are congenial for nodule development and symbiotic nitrogen fixation, high concentrations of soil nitrate inhibit nodule development and functioning. For instance, within 48 hours of treatment with NH_4NO_3, a collapse of meristematic activity in the nodule and changes in sub-cellular structure of infected cells are observed [2]. A prolonged exposure to NH_4NO_3, inhibits the formation of infection threads and nodule primordia that together result in a decrease in nodule number [3]. Although the molecular mechanism underlying nitrate-triggered inhibition of nodule initiation and development is not known, the inhibitory effect is proposed to be indirect [3]. Indeed, nitrate inhibition of nodulation is overcome by the ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG) [4], suggesting that the nitrate-inhibition of nodulation could be mediated by ethylene. In addition to the negative effects of nitrate on nodule initiation and development, short-term treatment with high nitrate also results in 80% decline in nitrogen fixation activity and nodule senescence [5-7]. However, in spite of this data, the mechanistic insight to the inhibitory effects of nitrate on nodule initiation and development is unknown.

Medicago truncatula is an excellent model to study legume-rhizobium symbiosis. A *M. truncatula* nodule can be distinguished into four zones along the longitudinal axis; meristem, infection zone, fixation zone and senescence zone. The meristem persistently adds new cell layers to the nodule and is the most distal zone from the root. The meristem is followed by an infection zone were the invading bacteria are internalized in the meristematic cells that just stopped dividing. During the process of internalization, the rhizobia are engulfed in a host-derived membrane compartments that gain a new organelle-like identity. These transient organelles are called symbiosomes. As the nitrogen-fixing capacity of

symbiosomes ceases over time, the infected cells senesce and the symbisomes gain a lytic identity. This phenotype is first visible in the cell layers most proximal to the root.

Symbiosomes are an example of a membrane delimited sub-cellular compartment. Membrane-bound sub-cellular compartments, e.g. organelles, are a hallmark of eukaryotic cells. Each sub-cellular organelle provides the requisite functional and spatial ambience for compartment-specific biochemical reactions. For instance, symbiosomes provide the necessary microaerobic environment for the Nitrogenase enzyme complex, which catalyzes symbiotic nitrogen fixation. The establishment and maintenance of such membrane compartmentalization requires transport of cargo within the endomembrane system. The endomembrane system is elaborate and includes endoplasmic reticulum, the Golgi apparatus, plasma membrane and vacuoles. The cargo is transported within the endomembrane system by small, spherical, membrane-delimited vesicles. These vesicles facilitate the anterograde and retrograde transport as well as endo- and exocytosis of cargo [8, 9]. The fusion of the vesicle and target membrane is energetically unfavorable process. To overcome the energy barrier and facilitate fusion of vesicle and target membranes, eukaryotes use SNARE (soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptors) proteins. SNARE proteins are classified based on their function and sub-cellular localization. SNAREs associated with vesicles are called v-SNAREs, whereas those associated with the target-membrane are called t-SNAREs. Generally, v-SNAREs possess a conserved arginine in the center of SNARE domain and are designated as VAMPs (vesicle-associated membrane proteins) [8, 9]. In plants, VAMP72 proteins mediate vesicle fusion associated with exocytosis and were shown to be important for plant interaction with pathogenic as well as symbiotic microbes [10-12].

VAMP72 genes form a small family that can be divided into 4 putative orthology groups [10]. One of the groups includes the *M. truncatula MtVAM721d* and *MtVAMP721e* genes, which function in the intracellular infection process of rhizobium and endomycorrhizal fungi [10]. This function might be specific, as *Arabidopsis thaliana* (Arabidopsis) that is unable to establish an endomycorrhizal symbiosis, lost its putative orthologous gene in this group [10]. Studies in Arabidopsis suggest that other *VAMP* genes perform functions that are more generic; e.g. *AtVAMP727* functions in protein transport to storage vacuoles [13], whereas *AtVAMP721* and *AtVAMP722* are essential in cell plate formation [14]. Interestingly, *AtVAMP721/722* are also co-opted in plant pathogen response to target vesicles containing cell wall–reinforcing material to the plasma membrane at sites of fungal attack [11]. *M.*

truncatula has 3 putative orthologs of *AtVAMP721/722*; named *MtVAMP721a*, *MtVAMP721b* and *MtVAM721c* [10]. Recently, it is found that the silencing of *MtVAMP721d* and *MtVAMP721e* and derepression of *MtVAMP721a* adversely affects symbiosome development [10, 12]. Repression of *MtVAMP721a* is mediated by MtRSD, a Cysteine-2/Histidine-2 type (C2H2) transcription factor and an *Mtrsd* knock mutant showed defects in differentiation of symbiosomes [12].

Besides *MtRSD*, several other transcription factors regulating early nodule development have been characterized e.g. *NSP1, NSP2, NIN, EFD, HAP2.1, ERF1* and *ERN* [For recent reviews, see 15]. Likewise, a transcriptional regulator of nodule senescence is known; namely *MtNAC969*, which is a member of NAC (NAM, ATAF, CUC) family, and shown to regulate nodule premature senescence and abiotic stress tolerance in *M. truncatula* [16]. In this study, we have functionally characterized a novel NAC transcription factor, *MtNAC074*, which is strongly induced in *M. truncatula* nodules upon nitrate treatment. Genetic and chemical interference with ethylene biosynthesis and signaling revealed ethylene as nitrate-induced secondary messenger required for *MtNAC074* expression. MtNAC074 is a putative transcriptional repressor, which targets *VAMP721a-c* and retards symbiosome development.

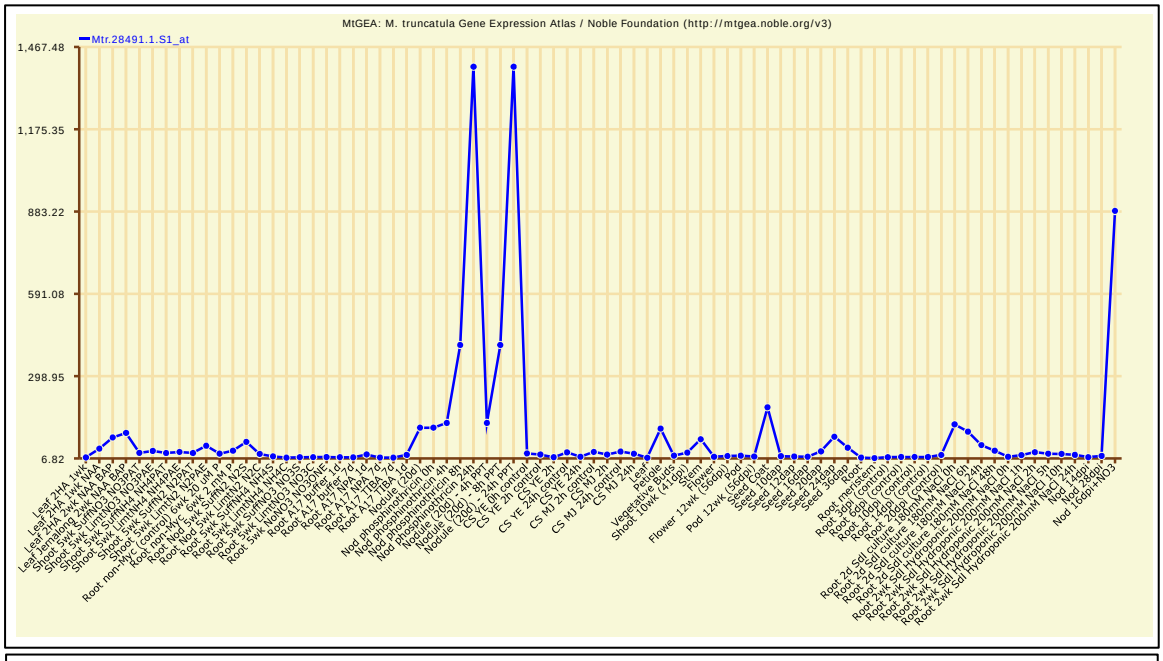

Figure 1: Transcript profile of *MtNAC074* / Medtr1g069805 (probe id. Mtr.28491.1.S1_at) in *M. truncatula* Gene Atlas in various tissues and in response to chemical treatments.

Results

MtNAC074 is highly induced in nodules upon nitrate treatment

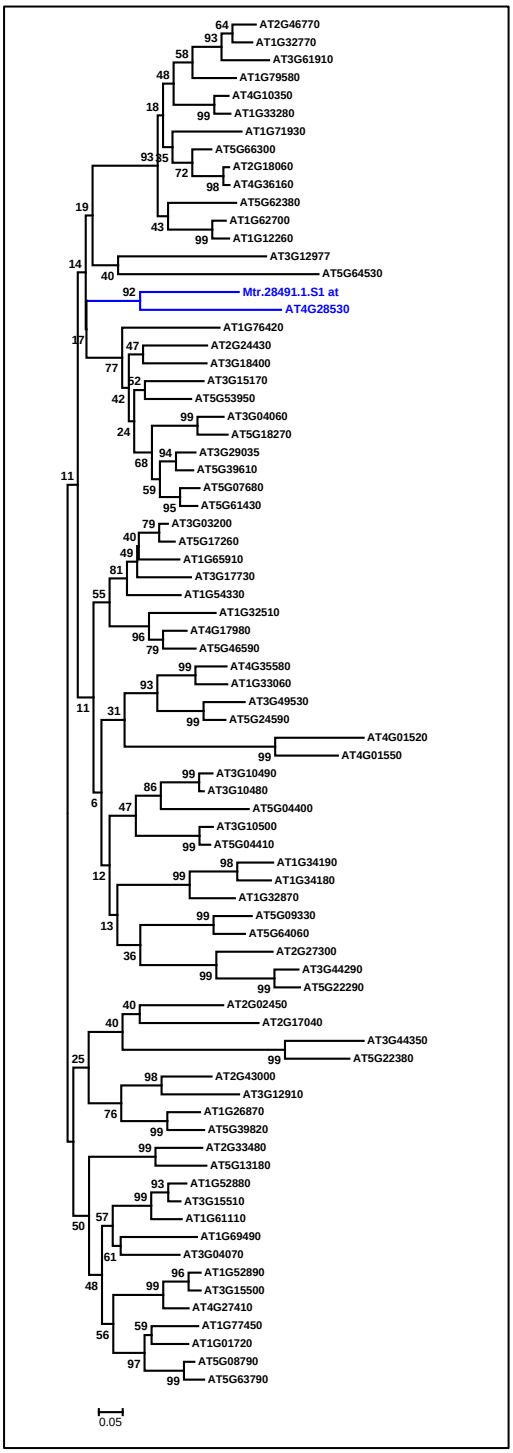

Figure 2: Topology of an unrooted neighbor- joining phylogeny constructed using full-length NAC domain sequences of *A. thaliana NAC* genes and MtNAC074 protein sequence. Branch support was obtained from 2,000 bootstrap repetitions.

Aiming to identify key transcriptional regulators that convert the nitrate induced root nodule senescence response; we exploited the *M. truncatula* Gene Expression Atlas (MtGEA) [17]. In the MtGEA, we analyzed the expression profile of all the 1,478 transcription factors annotated on the GeneChip in root and nodule samples, including the transcriptomes of nodules treated with NH_4NO_3 (see Chapter 2). From this data set, we identified a NAC transcription factor with gene ID Medtr1g069805 (probeset Mtr.28491.1.S1_at) that was highest induced in nodules 48 hr post exogenous nitrate application (Fig. 1). However, based on MtGEA expression profile of this NAC, while the nitrate-dependent upregulation is nodule specific, it is also expressed in other non-nitrate treated tissues e.g. hypocotyl, stem and root. Comparative analysis and phylogenetic reconstruction revealed that the encoded protein is highly homologous to Arabidopsis *AtNAC074* (At4G28530) (Fig. 2). Hence, the *M. truncatula* gene was named *MtNAC074*. To validate the nitrate-responsiveness of *MtNAC074* in nodules, *M. truncatula* plants harboring young nodules (2 week post inoculation) were treated with 15 mM NH_4NO_3 for 24 hrs. [18]. Subsequently, RNA was isolated from roots and nodules and used to monitor *MtNAC074* expression by qRT-PCR. This revealed a ~33 fold upregulation of *MtNAC074* in nitrate treated nodules, compared to untreated nodules (Fig. 3A). In agreement with MtGEA expression profiles, we noted that *MtNAC074* is also expressed in the root of the

plant, though nitrate-dependent induction of *MtNAC074* is not observed in roots (Fig. 3A). This suggests that the nitrate-response of *MtNAC074* is nodule specific.

Nitrate-dependent induction of *MtNAC074* requires ethylene biosynthesis and signaling

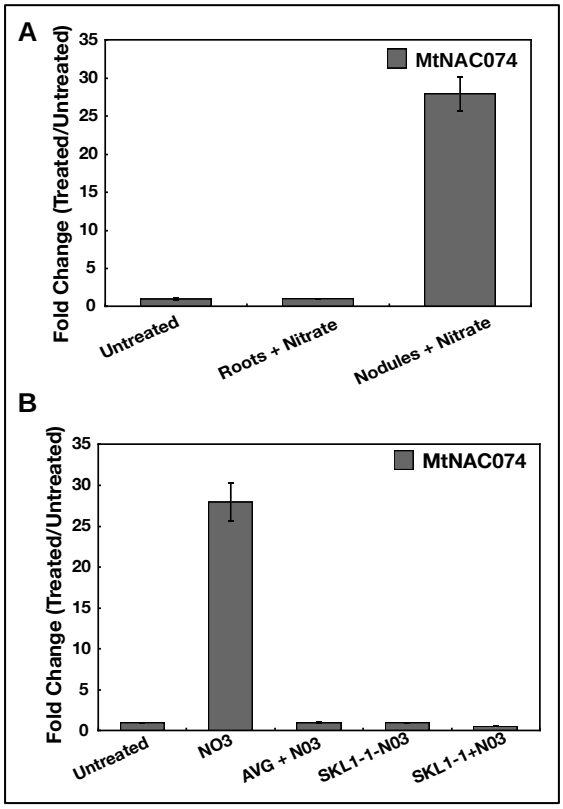

Figure 3. qRT-PCR based expression profile of nitrate dependent induction of *MtNAC074* in (A) nodules and, (B) is requires ethylene biosynthesis and signaling.

Nitrate triggered ethylene biosynthesis was proposed as a possible mechanism of inhibition of nodulation in alfalfa (*Medicago sativa*) [19]. Hence, we tested whether ethylene biosynthesis and signaling is also required for the nitrate-dependent *MtNAC074* expression. To this end, we pre-treated 2 wpi *M. truncatula* plants for 24 hours with 1 µM of the ethylene biosynthesis blocker AVG followed by 24 hours treatment with 15 mM NH_4NO_3 (AVG + N). In addition, 2 wpi nodulating plants treated with 15 mM NH_4NO_3 for 24 hrs (N24) and untreated plants were also included in the experimental design. Furthermore, to test the role of ethylene signaling, we used the ethylene signaling mutant sickle (*skl*) that harbors a knockout allele of *ein2* [20]. *skl-1/ein2* mutant plants were treated with or without 15 mM NH_4NO_3 for 24 hours. Using qRT-PCR, the expression of *MtNAC074* in root nodules was compared in each treatment. As described above *MtNAC074* was ~30x induced in nodules of wild type plants treated with 15 mM NH_4NO_3. However, no such induction could be found in nodules if plants were pretreated with AVG, nor in nodules of nitrate treated *skl-1/ein2* mutant (Fig. 3B). This suggests that *MtNAC074* nitrate-dependent upregulation requires ethylene biosynthesis and signaling.

Ectopic expression of *MtNAC074* affects symbiosome development

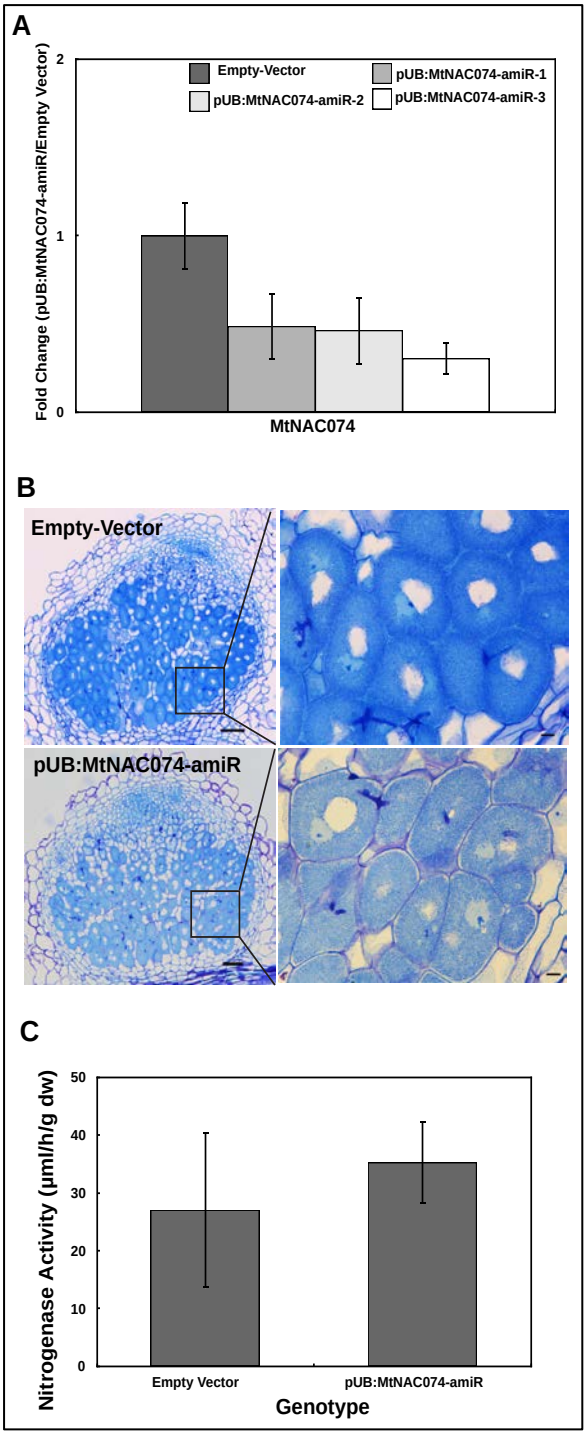

Figure 4: Artificial microrna mediated knockdown of *MtNAC074*. (A) Knockdown in three independent nodulating roots showing upto 80% silencing. (B) Toluidine stained longitudinal sections of *pUB:MtNAC074-amiRNA* and Empty Vector control nodules. (C) Acetylene reduction assay comparing the Nitrogenase activity of Empty Vector control and *pUB:MtNAC074* nodules.

High concentrations of exogenous nitrate has a dual affect on leguminous plants – it inhibits nodule initiation and development as well as it triggers nodule senescence [18]. Several transcription factors with opposing effects have been identified that control nodule senescence. Exogenously applied fixed nitrogen triggers premature nodule senescence within 24-48 h. As this timeframe coincides with the transcriptional induction of *MtNAC074* we aimed to determine whether also MtNAC074 has a function in nodule senescence. To do so, two experiments were conducted; ectopic expression and knock down of *MtNAC074* expression using *Agrobacterium rhizogenes* mediate transformation. This transformation procedure generates compound plants with transgenic roots and non-transgenic shoots. For knocking down *MtNAC074* expression we used an artificial microRNA (amiRNA). amiRNA based gene silencing has been successfully used in *M. truncatula* to study gene function [21]. The *MtNAC074amiR* construct was placed under control of the *A. thaliana* At*UBQ3 (Ubiquitin 3)* promoter, which is highly active in *M. truncatula* roots and nodules [22]. Similarly, for ectopic expression we used the same promoter. Compound plants were generated harboring transgenic roots that harbor either *pUBQ3::MtNAC074amiR* or *pUBQ3::MtNAC074*. In both cases roots had

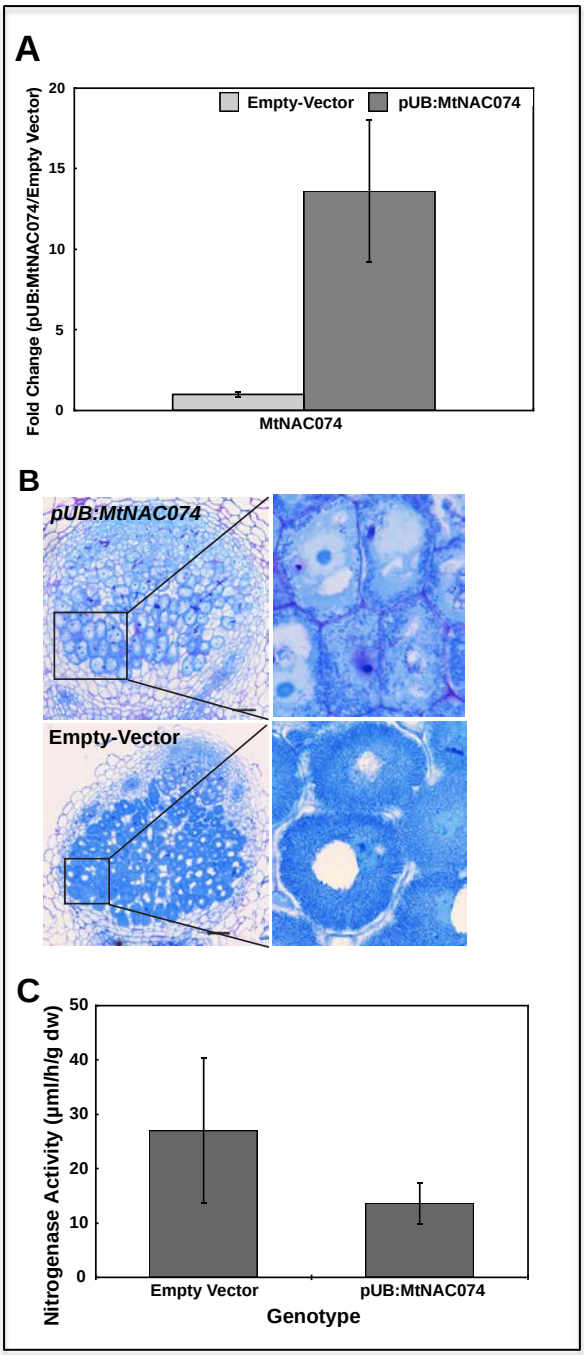

Figure 5. Symbiosome development is affected by ectopic expression of MtNAC074 (A) *pUB:MtNAC074* whereas (B) Empty vector nodules showing normal symbiosome development. (C) Acetylene reduction assay depicting reduced Nitrogenase activity in *pUB:MtNAC074*, Empty vector control and *pUB:MtNAC074-amiR* nodules ($p < 0.05$)

a normal appearance and were inoculated with *Sinorhizobium meliloti* to initiate nodulation. Nodules were harvested 2 weeks post inculcation. First, we determined *MtNAC074* expression levels in transgenic nodules by qRT-PCR. This revealed a silencing level of *MtNAC074* of up to 80% (Fig. 4A). Next, we compared the sub-cellular morphology of the transgenic nodules with nodules formed on control roots. Toluidine Blue stained longitudinal cross sections revealed no obvious phenotype in *MtNAC074* knock down nodules (n=20) (Fig. 4B). However, we observed that all transgenic nodules (n=20) expressing *pUBQ3::MtNAC074* displayed 15x upregulation of *MtNAC074* transcript (Fig. 5A), which was accompanied by a severe retardation in symbiosome development, loss in radial arrangement of symbiosomes and cells incompletely filled with symbiosomes (Fig. 5B). These defects in nodule development were absent in the *pUBQ3::* Empty (empty vector) control nodules, which showed cells displaying fully developed symbisomes that are packed in the characteristic radial pattern (Fig. 5B). This suggests that the overexpression of *MtNAC074* directly or indirectly represses symbiosome development, and may hint to a function in premature nodule senescence.

Next, we questioned whether knockdown or ectopic expression of *MtNAC074* affects the nitrogen fixation efficiency. To confirm these findings an acetylene reduction assay was

performed on the *pUBQ3::*Empty*, pUBQ3::MtNAC074* and *pUBQ3::MtNAC074-amiR* to compare the nitrogenase activity of these transgenic nodules. In agreement with the overexpression and knockdown studies, the nitrogenase activity was comparable in *pUBQ3::*Empty and *pUBQ3::MtNAC074-amiR* [Fig. 4C]. In contrast, *pUBQ3::MtNAC074* displayed ~4x reduction in nitrogenase activity as compared to *pUBQ3::*Empty [Fig. 5C].

MtNAC074 represses MtVAMP721

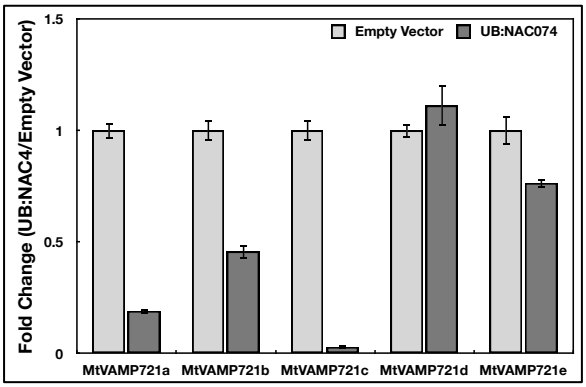

Figure 6: *MtVAMP721* expression in *pUB:MtNAC074* nodules based on qRT-PCR expression studies.

Exocytosis was shown to be required for normal symbiosome development in *M. truncatula* [10, 22]. Particularly, silencing the *MtVAMP721s*, which mediate exocytotic processes, impaired normal symbiosome development. *The M. truncatula* genome consists of 5 different MtVAMP721s; named *MtVAMP721a* to *MtVAMP721e*. Among these, silencing of *MtVAMP721d* and *MtVAMP721e* is shown to impair symbiosome development [10]. Similarly, repression of *MtVAMP721a* in the *Mtrsd* mutant coincides with impaired symbiosome development [12]. Hence, we hypothesized that overexpression of *MtNAC074* could repress the exocytotic pathway by targeting the members of *MtVAMP721* group. To this end, we tested the expression of all five *MtVAMP721* genes by qRT-PCR. Indeed, in *pUBQ3::MtNAC074* containing nodules, the expression of three *MtVAMP721* genes – namely *MtVAMP721a, MtVAMP721b* and *MtVAMP721c* (*MtVAMP721a-c*)- was reduced up to 20%, 40% and 90% respectively, whereas the expression of *MtVAMP721e* and *MtVAMP724d* was reduced up to ~30% (Fig. 6). Next, we questioned whether *MtNAC074* could repress *MtVAMP721a-c* expression by direct binding to the promoter region of these genes. To test this hypothesis, we first checked the promoters of *MtVAMP721a-c* (*pMtVAMP721a-c*) for the presence of putative NAC binding sites (PNBS). We found a PNBS (ANNNNNTCNNNNNNNCATGT), which was defined based on the NAC binding sites described earlier [23, 24], within *pMtVAMP721a-c* (Fig. 7A). The PNBSs are located between -3700 to -4250 bp for *MtVAMP721a*, -176 bp for *MtVAMP721b* and in between -1700 to -1800 for *MtVAMP721c*. The *in vitro* interaction of MtNAC074 with the PNBS within *pMtVAMP721a-c* was confirmed with an electrophoretic mobility shift assay (EMSA) (Fig. 7B). In addition, we investigated the *in vivo* MtNAC074-MtVAMP721a-c

interaction. To this end, we performed a chromatin immunoprecipitation-PCR (ChIP-PCR) on nodule tissues derived from pUBQ3::MtNAC074-GFP and anti-GFP antibodies. In the ChIP-PCR experiment, we observed ~3x enrichment of the PNBS of *MtVAMP721a-c* in the pUBQ3::MtNAC074-GFP nodule tissue as compared to wild type (Fig. 7C). Thus, based on EMSA and ChIP data, we conclude that MtNAC074 directly binds in the promoter region of *MtVAMP721a-c*.

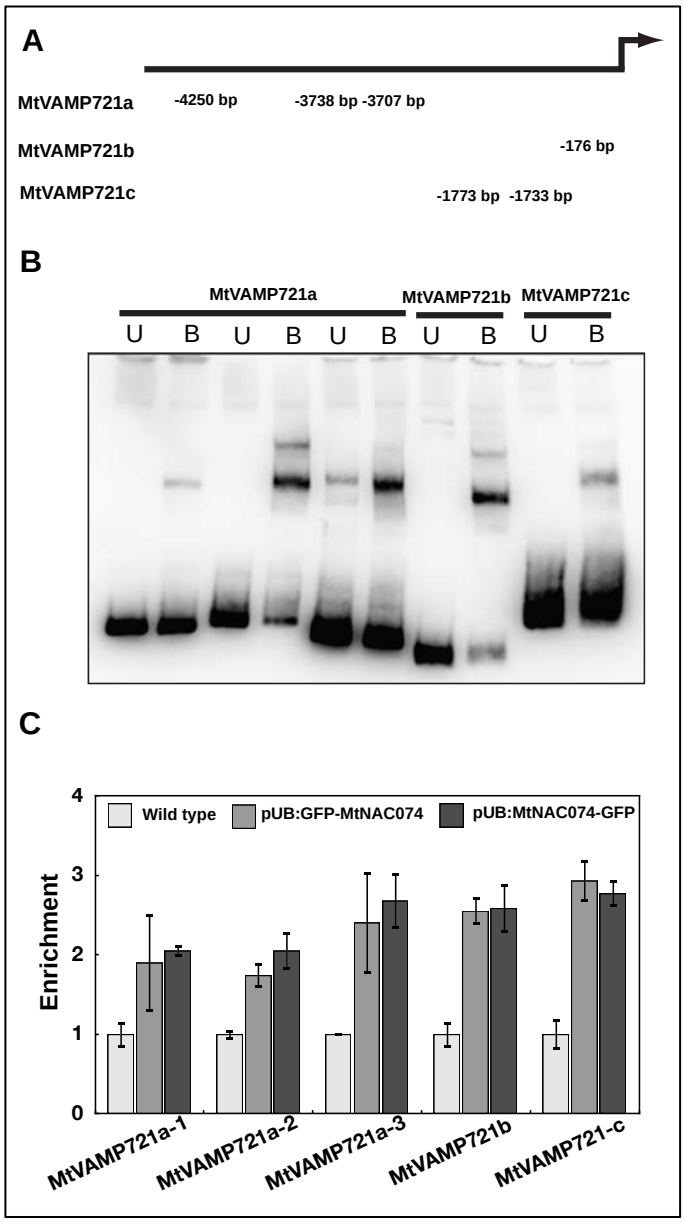

Figure 7: MtNAC074 directly binds to the promoter region of *MtVAMP721a-c*. (A) Putative NAC binding sites in the promoter regions of *MtVAMP721a-c*. (B) Electrophoretic mobility shift assay (EMSA) depicting unbound (U) or control (i.e. master mix + DNA fragment) and bound (B) probes of MtNAC074. (C) Chromatin immunoprecipitation-PCR with anti-GFP antibody performed on nodule tissue from *pUB:GFP-MtNAC074*, *pUB:MtNAC074-GFP* and wild type (A17) plants. Fold enrichment is calculated by comparing the chromatin immunoprecipitated DNA from *pUB:GFP-*

Discussion

In this study, we have identified a transcription factor, *MtNAC074*, which is induced in the nodules treated with inhibitory concentration (15 mM) of NH_4NO_3. Functional characterization of *MtNAC074* revealed that its nitrate-dependent expression requires the ethylene-SKL/EIN2

pathway. Ectopic *MtNAC074* expression interferes with symbiosome differentiation, and coincides with repression of *VAMP721a-c* expression. This latter could be a direct affect, as MtNAC074 can bind to the promoter regions of *MtVAMPa*, *b* and *c*. Taken together, we propose that a nitrate-ethylene-MtNAC074-MtVAMP72 regulatory chain is potentially involved in nitrate-induced inhibition of nodule development.

MtNAC074 **is a negative regulator of symbiosome development**

M. truncatula MtNAC074 is a member of the NAC family and a close homolog of the uncharacterized Arabidopsis gene *AtNAC074*. Our data indicate that MtNAC074 plays a role in the regulation of nitrate-induced retardation of symbiosome development. To this end, we show that - first, *MtNAC074* expression is induced upon nitrate treatment in nodules, but not in the roots. Second, overexpression of *MtNAC074* adversely affects symbiosome development, whereas the *MtNAC074* knockdown nodules are comparable to the empty vector controls. Third, acetylene reduction assay indicates nodules overexpressing *MtNAC074* have reduced nitrogenase activity as compared to empty vector controls. Fourth, MtNAC074 may directly target *MtVAMP721* genes, which are instrumental in the delivery of exocytotic cargo to the symbiosomes. Fifth, MtNAC074 is a potential repressor because *MtVAMP72*s expression is reduced in *MtNAC074* overexpressing nodules.

Nitrate arrests exocytotic delivery of cargo to symbiosomes

Our results indicate that MtNAC074 directly binds to the promoters of *VAMP721* genes, which regulate diverse processes in plants including cell plate formation, delivery of plant cargo to the interface of invading fungal haustorium as well as symbiosome and arbuscule formation [10, 11, 14]. Because qRT-PCR profiling suggests downregulation of *MtVAMP721a-e* in the nodules overexpressing *MtNAC074*, we hypothesize that MtNAC074 is a putative repressor of the *MtVAMP721* genes. Thus, it would be expected that nitrate-treatment, *MtNAC074* overexpression or reduced *MtVAMP721* levels could affect symbiosome development. Indeed, while nitrate-treatment was shown to retard membrane envelope development after the bacterial release into the host cells [2] and the silencing of *MtVAMP721d-e* genes blocks symbiosome formation [10]. *MtVAMP721a-c* are closely related to *VAMP721d,e* but belong to a different orthology group. Individual silencing of *VAMP721d* or *VAMP21e* does not lead to a phenotype. However, double silencing of *VAMP21d,e* displays defects in symbiosome development and bacterial release [10]. We

show that the overexpression of *MtNAC074* results in ~90% and ~30% reduction in levels of *VAMP721a-c* and *VAMP721d,e* mRNA respectively. Recently, it is found that a knockout mutant in the C2H2 transcription factor MtRSD affects symbiosome differentiation [12]. This phenotype coincides with a 20-50% reduction of *MtVAMP721a* expression, a gene that is a potential target of this transcription factor. As a homolog of MtVAMP721a in Arabidopsis not only was found to control cell plate formation, but also controls resistance response to invading pathogens it was hypothesized that active MtVAMP721a repression by MtRSD is essential for symbiosome formation [11]. In such model, MtVAMP721a acts as negative regulator of symbiosome formation. Our finding that *MtVAMP721a*, as well as other *MtVAMP721* genes, are repressed due to ectopic expression of MtNAC074, which coincides with defects in symbiosome differentiation, suggests a positive role of MtVAMP721 genes in symbiosome formation. Such positive role for *MtVAMP721* genes is in agreement with the silencing studies on *MtVAMP721d* and *MtVAMP721e*, which also showed defects in symbiosome differentiation [9]. Similar studies on MtVAMP721a-c could reveal the precise functioning of these genes in this process.

Ethylene is nitrate-induced second messenger inhibiting nodule development and function

Ethylene plays a critical role in nodule initiation, development and senescence. Exogenously applied ethylene inhibited nodule initiation in *P. sativum* [25] whereas the ethylene signaling mutant *ein2/skl* resulted in hypernodulation in *M. truncatula* and *Lotus japonicus* [26, 27]. Ethylene was shown to exert its inhibitory effect on nodulation by repressing early plant responses after nod factor perception but before calcium spiking [28]. Interestingly, marked increase in 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase activity and ethylene production rates was also observed within 24 hours of 10 mM NO_3^- treatment [19]. These inhibitory effects of nitrate-induced ethylene are blocked upon chemically blocking ethylene biosynthesis [4]. In agreement with these chemical studies, using *skl/ein2* mutant, we provide genetic evidence that nitrate-induced inhibition of nodule development is mediated by ethylene.

Materials and Methods

Plant materials, growth conditions and transformation

Medicago truncatula accession Jemalong A17 plants were grown in a growth chamber with 20°C and 16-hr day/8-hr night cycle. One week old plants were infected with 2 ml (OD_{600} 0.1) transgenic *Sinorhizobium melilotii* strain 2011 to induce nodulation. For nitrate treatment experiments, 21 dpi plants were transferred to fresh perlite mixed with 15 mM NH_4NO_3 in liquid Fahraeus medium. The nodules were harvested at 24 hours after nitrate treatment. For untreated control nodules, plants were transferred to perlite mixed with liquid Fahraeus medium without nitrate.

For generation of transgenic plants, *Agrobacterium rhizogenes*-mediated transformation of *M. truncatula* plants was performed using the protocol described in the Medicago Handbook (http://www.noble.org/MedicagoHandbook/pdf/AgrobacteriumRhizogenes.pdf). Transgenic plants were screened based on DsRED fluorescence, transplanted in perlite and grown as described above.

Constructs and plasmids

MtNAC074 open reading frame was amplified, using Phusion high fidelity Taq polymerase (New England Biolabs), with or without the stop codon for EMSA and overexpression studies respectively. For the PCR amplification, the cDNA of 21 dpi nodules was used and the amplicons were directionally cloned into pENTR TOPO-D (Invitrogen). The *MtNAC074* cassettes weres recombined in the Gateway destination vector with *Arabidopsis UBQ3* promoter created by digesting pK7FWG2 with *HindIII-SpeI* as described by Limpens *et al.,* (2009) [22] and carrying a DsRED expression marker in the backbone to allow for selection of transgenics [29].

RNA isolation, cDNA synthesis and qRT-PCR

RNA was isolated from 21 dpi nodules followed by an on-column digestion. cDNA synthesis was performed, as instructed in the supplier's manual, on 1 µg total RNA using Iscript RT-PCR kit (Biorad) in 20 µl reaction volume. The qRT-PCR profiling was done using three

technical replicates using 4 ul of the diluted (1:50) cDNA sample. The qRT-PCR reaction was performed on a MyIQ5 (Biorad) (40 cycles of 95°C for 10 s and 60°C for 1 min).

Acetylene Reduction Assay (ARA)

ARA was performed by modifying the protocol described by Oke et al., (1999) [30]. Briefly, 3 nodulating roots of *M. truncatula* plants were excised and placed in a glass vial sealed with an air-tight cap. From the vials, 1 ml air was removed before injecting with 1 ml acetylene. The nodulating roots + acetylene was incubated for 60 minutes at ambient temperature. The ethylene production was measured using a gas chromatograph (Chrompack Packard, model 438 with Flame Ionizator Detector) equipped with a column of Porapak (80/100 mesh). After the measurement, the nodules were excised to determine fresh weight.

Phylogenetic Analysis

Phylogenetic analysis was performed using the neighbour-joining (NJ) analysis on protein sequences downloaded from www.phytozome.net. The NJ-analysis for NACs was performed using full-length protein sequence of MtNAC074 and all the *Arabidopsis thaliana* NACs. The protein sequences were aligned using the Clustal W option in MEGA 5.0 by applying Blosum62 matrix, a gap open penalty of 12 and a gap extension penalty of 3. An unrooted phylogenetic tree was reconstructed using neighbor-joining approach and 2,000 bootstrap repetitions to evaluate statistical support of the branches.

Electrophoretic Mobility Shift Assay (EMSA) and Chromatin Immunoprecipitation (ChIP)

EMSA [31, 32] experiments were performed as described earlier. Briefly, pENTR MtNAC074-stop TOPOD vector, harboring the *MtNAC074* coding sequence with the stop codon, was recombined with a destination vector with ampicillin resistance and derived from pSPUTK, an in vitro transcription/translation vector (Strategene). The protein was expressed by in vitro transcription/translation with TNT SP6 High-Yield Wheat Germ Protein Expression system as per manufacturer's protocol (Promega). The promoter fragments of *MtVAMP721a, b* and *c* were amplified from *M. trunctula* genomic DNA and cloned using TA-cloning in pGEM-T-Easy vectors. These cloned promoter fragments were used to generate DNA probes by PCR. The MtNAC074 protein (2 µl from the *in vitro* reaction) was incubated

for 1 hr on ice with 40 fmol of biotinylated DNA probe in binding buffer (1 mM EDTA, pH 8.0, 7 mM HEPES pH7.3, 0.35 mg/ml BSA, 0.7 mM DTT, 60 µg/ml salmon sperm DNA, 1.3 mM spermidine, 2.5% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 8% glycerol). The DNA-protein complexes were resolved on a 5% native SDS-PAGE gel. After electrophoresis, the gel was blotted to Amersham Hybond-N^+ membrane. The signal was detected from the blotted membrane using the chemiluminescent nucleic acid detection kit (Pierce Chemical Co.).

ChIP as performed as described earlier [33]. Briefly, for IP we used an anti-GFP antibody (Clonetech). We harvested 2g nodulating roots from 4 weeks old plants. The tissue was infiltrated with 37 ml 1% formaldehyde buffer [0.4 M sucrose, 10 mM Tris-HCl (pH 8.0), 1 mM PMSF, 1 mM EDTA] for 30 min under vacuum at 25 psi. All the tissue was snap-frozen in liquid nitrogen. Two biologically independent replicates where each replicate was derived from MtNAC074 tagged with GFP at the C- and N-terminal were used for the ChIP. The ChIPed DNA was tested for enrichment using qRT-PCR.

References

1. Oka-Kira E, Kawaguchi M: **Long-distance signaling to control root nodule number**. *Current opinion in plant biology* 2006, **9**(5):496-502.
2. Dart PJ, Mercer FV: **The influence of ammonium nitrate on the fine structure of nodules of medicago tribuloides Desr. and trifolium subterraneum L**. *Archives of Microbiology* 1965, **51**(3):233-257.
3. Malik NSA, Calvert HE, Bauer WD: **Nitrate Induced Regulation of Nodule Formation in Soybean**. *Plant physiology* 1987, **84**(2):266-271.
4. Ligero F, Caba JM, Lluch C, Olivares J: **Nitrate Inhibition of Nodulation Can Be Overcome by the Ethylene Inhibitor Aminoethoxyvinylglycine**. *Plant physiology* 1991, **97**(3):1221-1225.
5. Bisseling T, Vandenbos RC, Vankammen A: **Effect of Ammonium-Nitrate on Synthesis of Nitrogenase and Concentration of Leghemoglobin in Pea Root-Nodules Induced by Rhizobium-Leguminosarum**. *Biochimica et biophysica acta* 1978, **539**(1):1-11.
6. Streeter JG: **Nitrate Inhibition of Legume Nodule Growth and Activity .2. Short-Term Studies with High Nitrate Supply**. *Plant physiology* 1985, **77**(2):325-328.
7. Chen PC, Phillips DA: **Induction of Root Nodule Senescence by Combined Nitrogen in Pisum sativum L**. *Plant physiology* 1977, **59**(3):440-442.
8. Lipka V, Kwon C, Panstruga R: **SNARE-Ware: The role of SNARE-Domain proteins in plant biology**. *Annual review of cell and developmental biology* 2007, **23**:147-174.
9. Bassham DC, Blatt MR: **SNAREs: Cogs and coordinators in signaling and development**. *Plant physiology* 2008, **147**(4):1504-1515.
10. Ivanov S, Fedorova EE, Limpens E, De Mita S, Genre A, Bonfante P, Bisseling T: **Rhizobium-legume symbiosis shares an exocytotic pathway required for arbuscule formation**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(21):8316-8321.
11. Kwon C, Neu C, Pajonk S, Yun HS, Lipka U, Humphry M, Bau S, Straus M, Kwaaitaal M, Rampelt H *et al*: **Co-option of a default secretory pathway for plant immune responses**. *Nature* 2008, **451**(7180):835-U810.
12. Sinharoy S, Torres-Jerez I, Bandyopadhyay K, Kereszt A, Pislariu CI, Nakashima J, Benedito VA, Kondorosi E, Udvardi MK: **The C2H2 Transcription Factor REGULATOR OF SYMBIOSOME DIFFERENTIATION Represses Transcription of the Secretory Pathway Gene VAMP721a and Promotes Symbiosome Development in Medicago truncatula**. *The Plant cell* 2013, **25**(9):3584-3601.
13. Ebine K, Okatani Y, Uemura T, Goh T, Shoda K, Niihama M, Morita MT, Spitzer C, Otegui MS, Nakano A *et al*: **A SNARE complex unique to seed plants is required for protein storage vacuole biogenesis and seed development of Arabidopsis thaliana**. *The Plant cell* 2008, **20**(11):3006-3021.
14. Zhang L, Zhang H, Liu P, Hao H, Jin JB, Lin J: ***Arabidopsis* R-SNARE Proteins VAMP721 and VAMP722 Are Required for Cell Plate Formation**. *PloS one* 2011, **6**(10):e26129.
15. Oldroyd GED, Murray JD, Poole PS, Downie JA: **The Rules of Engagement in the Legume-Rhizobial Symbiosis**. *Annual Review Genetics, Vol 45* 2011, **45**:119-144.
16. de Zélicourt A, Diet A, Marion J, Laffont C, Ariel F, Moison M, Zahaf O, Crespi M, Gruber V, Frugier F: **Dual involvement of a Medicago truncatula NAC transcription factor in root abiotic stress response and symbiotic nodule senescence**. *The Plant Journal* 2012, **70**(2):220-230.
17. Benedito VA, Torres-Jerez I, Murray JD, Andriankaja A, Allen S, Kakar K, Wandrey M, Verdier J, Zuber H, Ott T *et al*: **A gene expression atlas of the model legume**

Medicago truncatula. *The Plant journal : for cell and molecular biology* 2008, **55**(3):504-513.
18. Streeter J, Wong PP: **Inhibition of legume nodule formation and N2fixation by nitrate**. *Critical Reviews in Plant Sciences* 1988, **7**(1):1-23.
19. Caba JM, Recalde L, Ligero F: **Nitrate-induced ethylene biosynthesis and the control of nodulation in alfalfa**. *Plant, cell & environment* 1998, **21**(1):87-93.
20. Penmetsa RV, Uribe P, Anderson J, Lichtenzveig J, Gish JC, Nam YW, Engstrom E, Xu K, Sckisel G, Pereira M *et al*: **The Medicago truncatula ortholog of Arabidopsis EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations**. *The Plant journal : for cell and molecular biology* 2008, **55**(4):580-595.
21. Devers E, Teply J, Reinert A, Gaude N, Krajinski F: **An endogenous artificial microRNA system for unraveling the function of root endosymbioses related genes in Medicago truncatula**. *BMC plant biology* 2013, **13**(1):82.
22. Limpens E, Ivanov S, van Esse W, Voets G, Fedorova E, Bisseling T: **Medicago N2-fixing symbiosomes acquire the endocytic identity marker Rab7 but delay the acquisition of vacuolar identity**. *The Plant cell* 2009, **21**(9):2811-2828.
23. Wang X, Culver J: **DNA binding specificity of ATAF2, a NAC domain transcription factor targeted for degradation by Tobacco mosaic virus**. *BMC plant biology* 2012, **12**(1):157.
24. Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K: **Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter**. *The Plant cell* 2004, **16**(9):2481-2498.
25. Lee KH, Larue TA: **Exogenous Ethylene Inhibits Nodulation of Pisum-Sativum L Cv Sparkle**. *Plant physiology* 1992, **100**(4):1759-1763.
26. Penmetsa RV, Cook DR: **A Legume Ethylene-Insensitive Mutant Hyperinfected by Its Rhizobial Symbiont**. *Science* 1997, **275**(5299):527-530.
27. Nukui N, Ezura H, Minamisawa K: **Transgenic Lotus japonicus with an ethylene receptor gene Cm-ERS1/H70A enhances formation of infection threads and nodule primordia**. *Plant and Cell Physiology* 2004, **45**(4):427-435.
28. Oldroyd GED, Engstrom EM, Long SR: **Ethylene Inhibits the Nod Factor Signal Transduction Pathway of Medicago truncatula**. *The Plant Cell* 2001, **13**(8):1835-1849.
29. Limpens E, Ramos J, Franken C, Raz V, Compaan B, Franssen H, Bisseling T, Geurts R: **RNA interference in Agrobacterium rhizogenes-transformed roots of Arabidopsis and Medicago truncatula**. *Journal of experimental botany* 2004, **55**(399):983-992.
30. Oke V, Long SR: **Bacterial genes induced within the nodule during the Rhizobium–legume symbiosis**. *Molecular Microbiology* 1999, **32**(4):837-849.
31. Kaufmann K, Anfang N, Saedler H, Theissen G: **Mutant analysis, protein–protein interactions and subcellular localization of the Arabidopsis Bsister (ABS) protein**. *Molecular Genetics and Genomics* 2005, **274**(2):103-118.
32. Liu W, Kohlen W, Lillo A, Op den Camp R, Ivanov S, Hartog M, Limpens E, Jamil M, Smaczniak C, Kaufmann K *et al*: **Strigolactone biosynthesis in Medicago truncatula and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2**. *The Plant cell* 2011, **23**(10):3853-3865.
33. Kaufmann K, Muino JM, Osteras M, Farinelli L, Krajewski P, Angenent GC: **Chromatin immunoprecipitation (ChIP) of plant transcription factors followed by sequencing (ChIP-SEQ) or hybridization to whole genome arrays (ChIP-CHIP)**. *Nature Protocols* 2010, **5**(3):457-472.

Chapter 4

High-throughput transcriptome profiling of *Medicago truncatula* root nodules treated with ammonium nitrate

Authors: Vidyadhar Karmarkar[1,§], Nick Krom[2], Robin van Velzen[1], Erik Limpens[1], Ton Bisseling[1,3] and Rene Geurts[1]

Affiliations:

1. Laboratory of Molecular Biology, Wageningen University, Wageninengen, 6708PB , The Netherlands.

2. The Samuel Roberts Noble Foundation, Ardmore, OK, 73401, USA.

3. Department of Zoology, College of Science, King Saud University, Riyadh 11451, Saudi Arabia

FOOTNOTE:

§ Current Address: Max Planck Institute for Plant Breeding Research, Carl-von-Linne-Weg 10, 50829 Cologne, Germany.

Abstract

Exogenous soil nitrate inhibits symbiotic nitrogen fixation by affecting nodule initiation and development and triggering nodule senescence. Although these inhibitory effects of nitrate are known for almost a century, the underlying molecular mechanisms regulating the inhibition are unknown. Because transcription factors are important for interpreting exogenous and endogenous cues to trigger downstream responses, identifying nitrate induced transcription factors could provide a key to unravel the molecular basis of nitrate-induced inhibition of symbiotic nitrogen fixation. To identify nitrate-induced genes and transcription factors, we performed transcriptome profiling using RNA-Seq. We studied early transcriptome changes in 21 days post inoculation *Medicago truncatula* nodules treated with 15 mM NH_4NO_3. Using RNA-Seq, we interrogated three time points – 12, 18 and 24 hours post-nitrate treatment and an untreated control sample. The number of reads generated from the untreated and nitrate-treated time points ranged from 6.8 to 18.7 million. Following the transcriptome alignment on the *M. truncatula* genome, we used the transcriptome assembly to identify differentially expressed transcription factors. Based on transcriptome comparison, we identified 3,900 genes with fold change > 2x. In addition, in the top 50 upregulated transcription factors, we identified a preponderance of genes from the families NAC, WRKY, bZIP, bHLH, MYB and AP2-EREBP that were shown earlier to be associated with plant senescence. In summary, the current study has characterized the early changes in nitrate-induced perturbations in *M. truncatula* nodules. Our particular focus on differential expression of transcription factors, has led us to a list of lead candidates that can be functionally validated. However, the challenge is selection of lead candidates for functional validation. This will require either development of a high-throughput system for reverse-genetics, based on phenotypic screening using the available retrotransposon insertion lines of the transcription factors or an educated guess based on complementary knowledge. Given the conceptual and transcriptome similarity in leaf and nodule senescence described earlier, it is possible to select transcription factor candidates based on similarities in both processes.

Introduction

Exogenous nitrate is known for its negative effect on symbiotic nitrogen fixation in legumes for almost 100 years [1]. At concentrations of 10-20 mM, nitrate inhibits rhizobium infection, nodule initiation and growth [2]. Additionally, exogenous nitrates ceases nitrogen fixation in existing nodules that subsequently senesce [2]. In latter case lytic vesicles fuse with the endosymbiotic compartments that host the rhizobia (so-called symbiosomes), leading to terminal degradation of these symbiotic units. Nitrate-induced decline in nitrogen fixation and symbiosome degradation is evident already 24-36 hours post application [1]. This indicates the presence of dynamic responsive signaling networks to control the nitrogen balance of the plant in favor of nutrient uptake instead of the costly activity of nitrogen fixation. However little is known about how nitrate negatively affects nodule functioning and nitrogen fixation. We postulate that transcriptional networks that respond to environmental cues like exogenous nitrates form the key in the onset of premature nodule senescence.

RNA-Seq is a powerful high-throughput tool for transcriptome comparison [3]. RNA-Seq has a wide dynamic range to detect gene expression levels [3] and has been successfully used for characterizing the developmental transcriptome [4], alternative splicing and estimating abundance [5], RNA-editing [6] and quantitative analysis of transcriptomes [7]. Hence, we used an RNA-Seq approach to gain a preliminary insight to identify differentially expressed genes upon nitrate treatment. Among the differentially expressed genes, we aimed to identify early responsive transcription factors. Transcription factors regulate diverse processes ranging from plant development to physiology. They encode DNA-binding regulatory proteins that interact with the promoter and enhancer sequences. Such interactions facilitate activation or repression of target genes and enable the plant to respond to developmental, environmental and physiological cues. These external and internal signals are sensed and transduced by the plant to activate or repress transcription factors, which in turn activate or repress downstream regulatory programs. Hence, transcription factors are good nodes to search for key regulators of output pathways induced by various external and internal cues. In plants, transcription factors have also been deployed for abiotic and biotic stress tolerance [8, 9].

In legumes, only a limited number of transcription factors have been functionally characterized most of them involved in nodule initiation and development [10, 11]. [12]. Genome sequencing of the model legume *Medicago truncatula* identified over ~ 3,600

putative transcription factor genes [12]. However, to date only one transcription factor has been functionally characterized that affects nodule senescence [13]. *MtNAC969* expression is induced by nitrate treatment in the central zone of the nodule, similar as senescence markers like cysteine proteases. However, *MtNAC969* has a broader role, and controls root architecture and the susceptibility to saline stress as well. Such pleiotropic function hampers genetic dissection of the genetic networks controlling senescence.

We used the *M. truncatula* – *Sinorhizobium meliloti* system to study nitrate effects on nodule development and physiology. Nitrate affects nodule initiation and development as well as nodule senescence [1, 2, 13]. However, nitrate-induced transcription factors that control this process remain unknown. To gain an insight into the global changes in the nitrate-perturbed transcriptome of root nodules, we performed RNA-Seq and identified a set of nitrate-induced transcription factors, two of which have been functionally characterized in the following chapter of this thesis.

Results and Discussion

Biological assay for ammonium nitrate induced root nodule senescence

Treating nodules with 10-20 mM nitrate for 24 hours resulted in nodule senescence [1]. However, the exact nitrate-induced transcriptomic changes that could result in nodule senescence are unknown. The goal of this study was to characterize nitrate-induced transcriptome reprogramming and identify transcription factors that may mediate nitrate-induced inhibition of nodule development and onset of nodule senescence. Hence, as a first step we developed an assay to trigger root nodule senescence upon application of exogenous fixed nitrogen. Three weeks old plants that harbor root nodules were transferred to new perlite containing minimal Fahreus medium with 15 mM or 0 mM NH_4NO_3. The latter served as negative control. Nodules were harvested 12, 18 and 24 h post transfer. Cytohistochemical studies of these nodules (described in chapter 5) revealed first morphological signs of senescence 18h post transfer to 15mM NH_4NO_3 containing medium. A few infected cells most basal of the nodule contain degraded symbiosomes. This phenotype is more pronounced 24 h post transfer to NH_4NO_3 medium, were now in about 30-50% of cells symbiosome degeneration is visible. Hence, based on these data we conclude that root nodule senescence is activated in the first 18 h post NH_4NO_3 application.

Transcriptome changes associated to root nodule senescence

To characterize the transcriptome of *M. truncatula* root nodules RNA was extracted from nodules 0, 12, 18 and 24 hours post nitrate 15 mM NH_4NO_3 treatment. The integrity of the RNA was checked using a Bio-analyzer and confirmed on agarose gel analysis. The RNA was reverse transcribed to cDNA and sequenced with the Illumina Genome Analyzer II. To get an insight to the transcriptomic changes upon induction of root nodule senescence, we performed a single RNA-Seq experiment generating 10-20 million 100 bp reads per sample (Table 1). These reads were mapped to the *M. truncatula* annotated transcriptome (Mt. v3.5.3) using the alignment program TopHat [14]. The mapped reads were classified as either unique or non-unique depending upon if it mapped to only one or multiple reference sequence sites. Only 52-55% uniquely mapped to the annotated transcriptome, whereas only 1-2% were found to be not unique. As only ~55% of the sequence reads mapped to the annotated transcriptome, we also mapped the reads to the *M. truncatula* genome (Mt_v3.5). This revealed a very similar mapping percentage, suggesting an accurate annotation of the transcriptome. Therefore we argue that the remaining 40% of RNA-seq sequence reads either map to the non-sequence portion of the genome/transcriptome or, alternatively are of bacterial origin. The latter seems more likely as EST sequencing in *M. truncatula* was biased towards root nodules.

Table 1: RNA-seq data generated from nitrated treated nodules of *M. truncatula*.

	Untreated		N12		N18		N24	
Read size (bp)	100		100		100		100	
Number of reads	18755460		16871046		16422788		6817692	
Reads uniquely mapped to Medicago genome	10183531	(54.30%)	8803871	(52.18%)	8679089	(52.85%)	3555538	(52.15%)
Reads non-uniquely mapped to Medicago genome	1210756	(6.46%)	1223476	(7.25%)	1068379	(6.51%)	405991	(5.95%)
Reads uniquely mapped to Medicago transcriptome	10092818	(53.81%)	8733130	(51.76%)	8608729	(52.42%)	3525540	(51.71%)
Reads non-uniquely mapped to Medicago transcriptome	363540	(1.94%)	310410	(1.84%)	298081	(1.82%)	111247	(1.63%)
Reads uniquely mapped to Medicago transcription factors	209569	(1.12%)	177541	(1.05%)	188340	(1.15%)	83602	(1.23%)
Reads non-uniquely mapped to Medicago transcription factors	3054	(0.02%)	2550	(0.02%)	2572	(0.02%)	1111	(.02%)
Expressed transcripts	18941		18419		18533		15581	
Expressed annotated transcripts	18162	(95.89%)	17738	(96.30%)	17820	(96.15%)	15120	(97.04%)
Expressed un-annotated transcripts	779	(4.111%)	681	(3.70%)	713	(3.85%)	461	(2.96%)

For measuring the transcript counts, we used the Fragments per kilobase of exon per million fragments mapped (FPKM) method [15]. For our study, we considered any gene with FPKM value > 0 as transcriptionally active. The number of expressed transcripts ranged from 15,581 to 18,941. Of these transcripts, 96±1% was functionally annotated. First, we focused on the transcriptional differences between untreated nodules and nodules that where exposed to exogenous nitrogen for 24h. At this time-point, nodule senescence is induced and about 30-50% of infected cells displayed symbiosome degeneration. This process is associated with massive transcriptional reprogramming and a total of ~3,000 genes can be

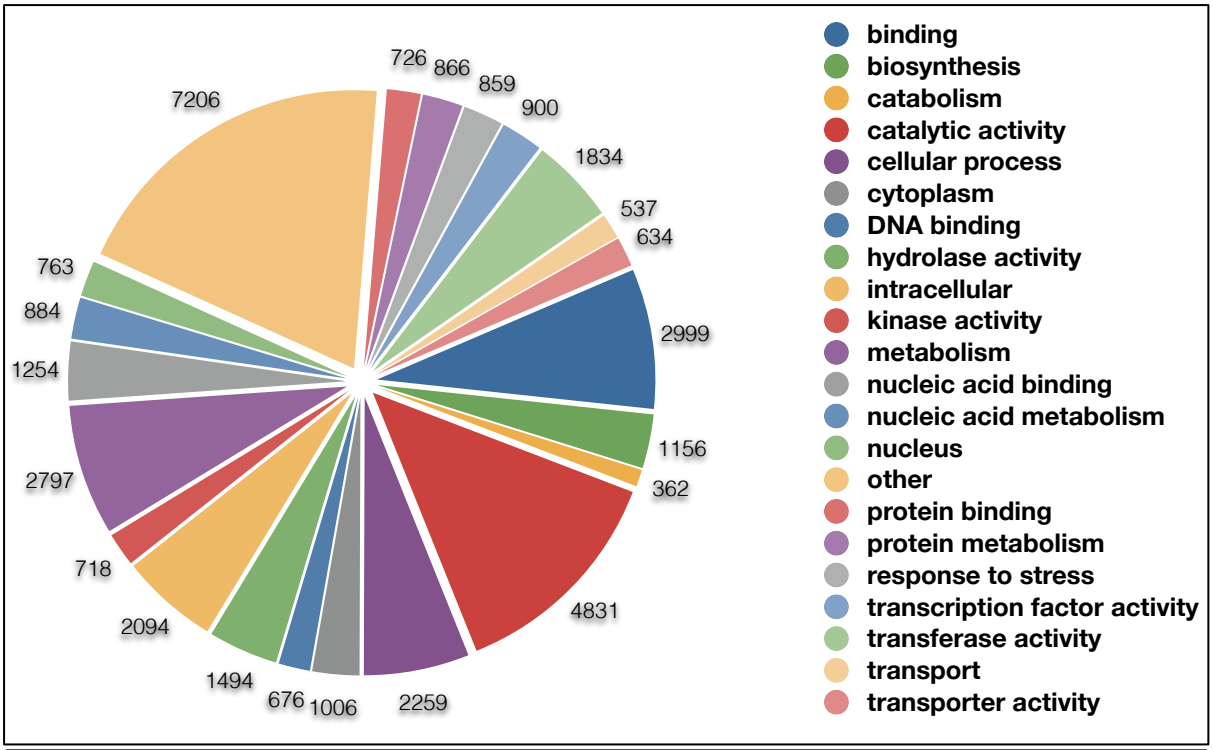

Figure 1. Gene Ontology Term Analysis of genes upregulated upon treatment of *M. truncatula* nodules with 15 mM NH_4NO_3.

identified with an expression change ≥ 2 fold. Among these, are genes that are involved in senescence-associated processes e.g. protein degradation, metabolism, stress, kinase activity (Figure 1). As we were interested in transcriptional changes prior to onset of senescence, we first determined the temporal dynamics of these 'senescence-associated genes'. This revealed that most genes show strongest expression 24 h post NH_4NO_3 treatment. However, transcriptional activation of these genes occurred already as early as 12 h post treatment, suggesting that root nodule senescence is induced within this timeframe.

Next, we focused on the earliest time point in the expression study; 12 h post NH_4NO_3 application. A total of ~3,900 genes display a 2-fold expression difference at this time point. In the top 50 upregulated transcription factors, based on the fold change, we identified a preponderance of the transcription factor families have been described to be associated with stress and leaf senescence [16]. For instance, bHLH (b-helix loop helix), NAC (NAM, ATAF, CUC), MYB (Myeloblastosis), bZIP (basic leucine zipper), AP2-EREBP (Apetala2-Ethylene Response Element Binding Protein), WRKY (WRKY domain protein) and Zinc fingers. The members of each of these families were either upregulated only in samples treated with 12 hours of nitrate or in all three treatments (Figure 2). For 26 candidates, we observed a

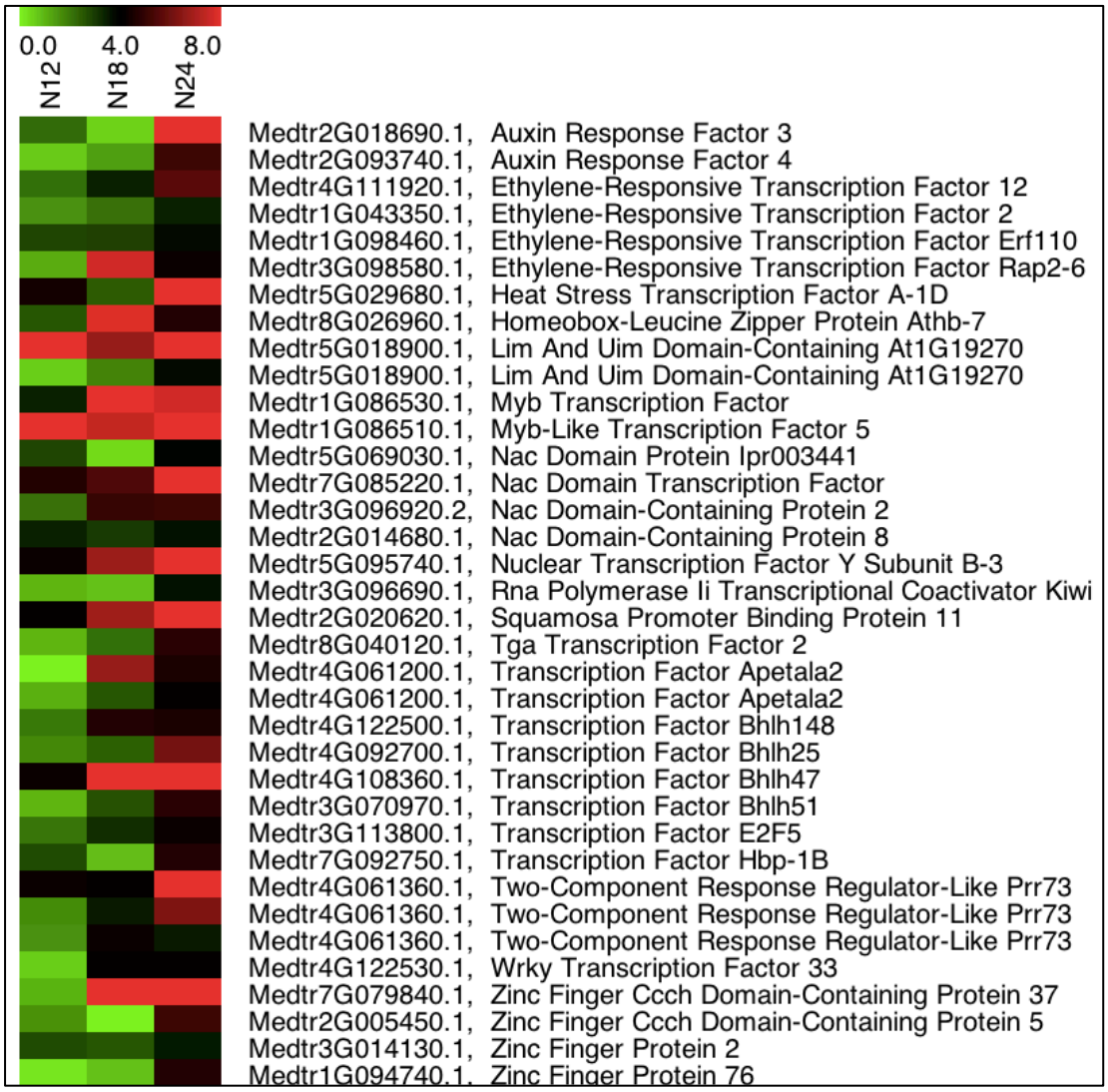

Figure 2. Top 35 transcription factors upregulated after 24 hour treatment with15 mM NH_4NO_3.

steady increase in fold change values with the duration of nitrate treatment. The lead candidates for functional validation described in the next chapters were selected from this list of 26 candidates.

Next we aimed to validation the RNA-Seq data by using qRT-PCR, we revalidated the expression trend of randomly selected candidate genes from different senescence-associated transcription factor families [16], namely bZIP (Medtr8g040120.1), NAC (Medtr3g096920.1), CCAAT (Medtr5g095740.1), MYB1 (Medtr1g086510.1), MYB2 (Medtr1g086530.1), AP2-EREBP (Medtr4g061200.1) and bHLH (Medtr4g092700.1). We performed qRT-PCR on an independent biological replicate of untreated and nitrate-treated nodules. As shown in figure 3, among the genes that were tested, we observed increase in

transcript levels with time. In table 1, we present summary of fold changes observed in RNA-Seq and qRT-PCR for the genes subject to revalidation. Thus, we observed a similar expression trend in the revalidation studies that confirms the robustness of our RNA-Seq experiment.

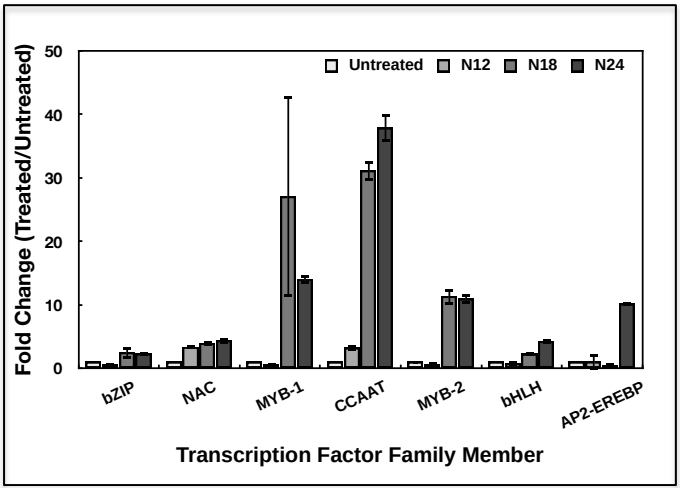

Figure 3. qRT-PCR revalidation of randomly selected genes from RNA-Seq studies. bZIP (Medtr8g040120), NAC (Medtr3g096920.1), MYB1 (Medtr3g096920.1), CAAT (Medtr5g095740.1), MYB2 (Medtr1g086530.1), bHLHP (Medtr4g092700.1) and AP2-EREBP (Medtr4g061200.1).

Table 1. Fold change expression in root nodules of a selected set of transcription factors upon application 15 mM NH_4NO_3 (24 h). Expression levels have determined by RNAsed and qRT-PCR on independent biological samples.

	Fold Change	
TF ID	**RNA-Seq**	**qRT-PCR**
Medtr8g040120.1	4.75	3.36
Medtr3g096920.1	5.14	4.75
Medtr5g095740.1	9.07	38.29
Medtr1g086510.1	9.26	12.48
Medtr1g086530.1	7.66	10.18
Medtr4g061200.1	4.56	10.01
Medtr4g092700.1	6.1	4.66

Conclusion

In summary, the transcriptome data presented herein brings together early changes in *M. truncatula* nodules treated with 15 mM NH_4NO_3. We discovered that the transcription factor families upregulated after high concentration of nitrate and leaf senescence are similar. The transcription factors identified through differential gene expression analysis in RNA-Seq and qRT-PCR provide a list of candidates that can be tested for functional validation studies. Nevertheless, the next challenge is developing a method to shortlist lead candidate transcription factors for functional validation. Given the conceptual similarity in leaf and nodule senescence as well as similarities in their transcriptomes, it is possible to select lead candidates and transcription factor families for functional validation.

Material and Methods

Plant material and RNA isolation

M. truncatula Jemalong A17 seeds were germinated on Fahraeus medium plates. A week old healthy seedlings were transplanted on perlite and irrigated with Fahreus medium without nitrate. The plants were grown at 20°C and 16-hr day/8-hr night regime. At the time of transplanting, the plants were inoculated wit *Sinorhizobium melilotii* strain 2011. The plants were subsequently irrigated every week. At 21 days after inoculation, the plants were treated with 15mM NH_4NO_3 dissolved in liquid Fahraeus medium. The nodules were harvested at 12, 18 and 24 hours post-nitrate treatment. For untreated control samples, plants were irrigated with Fahraeus medium without nitrate.

E.Z.N.A. Plant RNA Isolation Kit (Omega Bio-Tek) was used to extract total RNA from the harvested nodules. The DNase treatment was done on column using Qiagen RNase-Free DNase followed by a second DNase treatment with Ambion® TURBO™ DNase. RNA quality was evaluated by gel electrophoresis, spectrophotometer and Agilent 2100 Bioanalyzer. The total RNA was checked for absence of genomic DNA contamination using intronic primers designed to amplify a 107 bp genomic fragment of Ubiquitin (TC10247intronF, 5'-GTCCTCTAAGGTTTAATGAACCGG-3'; TC102473 intronR, 5'-GAAAGACACAGCCAAGTTGCAC-3') (Kakar et al., 2008).

cDNA preparation and Illumina Sequencing

For the Illumina RNA-Seq, 5 different cDNAs were prepared from nitrate treated and untreated RNA samples. The libraries were prepared by the company Fasteris SA (Geneva, Switzerland) according to Illumina's instruction for mRNA-Seq protocols. The 3' adapter was the standard Illumina adapter whereas a barcode was inserted in the 5' RNA adapter. The sequencing was performed on Genome Analyzer II instrument.

Transcriptome analysis

We used a reference-based *ab initio* strategy [17] for mapping of the *M. truncatula* reads to the reference sequence. In our strategy, we first mapped the short-reads to the reference sequence using the TopHat splice-aware aligner at default settings [14]. We used the *M.*

truncatula reference genome (Mt. ver. 3.5) and transcriptome (Mt. ver. 3.5.3) as our reference sequence. The unmapped reads were either of low quality, from highly repetitive regions or unsequenced regions of the *M. truncatula* genome, *S. melilotii* sequences, or adapters/chimeric reads and were excluded from further analysis. In the second step, overlapping reads at each locus were grouped together to build a splice graph representing all putative transcript isoforms. Cufflinks [14] at default settings was used to traverse the graph to resolve isoform information.

The transcript and isoform expression was used for estimating digital gene expression (DGE) analysis. We used fragments per kilobase per million reads (FPKM) [15] to estimate to DGE counts. An expressed fragment corresponds to an FPKM value of ≥ 1. Such expressed fragments were classified as unique if it had only 1 best match with the reference sequence and as non-unique if it mapped to 2 or more locations on the reference sequence.

Quantitative real-time PCR validation

For qRT-PCR profiling, RNA was extracted from nodules 21 days after inoculation. The DNase digestion was performed on-column using the Qiagen RNase-Free DNase. cDNA synthesis was performed, as instructed in the supplier's manual, on 1 µg total RNA using Iscript RT-PCR kit (Biorad) in 20 µl reaction volume. The qRT-PCR profiling was done in triplicate on diluted (1:50) cDNA samples. The qRT-PCR reaction was performed on a MyIQ5 (Biorad) (40 cycles of 95°C for 10 s and 60°C for 1 min).

References

1. Streeter J, Wong PP: **Inhibition of legume nodule formation and N2fixation by nitrate**. *Critical Reviews in Plant Sciences* 1988, **7**(1):1-23.
2. Chen PC, Phillips DA: **Induction of Root Nodule Senescence by Combined Nitrogen in Pisum sativum L**. *Plant physiology* 1977, **59**(3):440-442.
3. Wang Z, Gerstein M, Snyder M: **RNA-Seq: a revolutionary tool for transcriptomics**. *Nature reviews Genetics* 2009, **10**(1):57-63.
4. Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG, van Baren MJ, Boley N, Booth BW *et al*: **The developmental transcriptome of Drosophila melanogaster**. *Nature* 2011, **471**(7339):473-479.
5. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L: **Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation**. *Nat Biotechnol* 2010, **28**(5):511-515.
6. Peng Z, Cheng Y, Tan BC, Kang L, Tian Z, Zhu Y, Zhang W, Liang Y, Hu X, Tan X *et al*: **Comprehensive analysis of RNA-Seq data reveals extensive RNA editing in a human transcriptome**. *Nature biotechnology* 2012, **30**(3):253-260.
7. Marguerat S, Schmidt A, Codlin S, Chen W, Aebersold R, Bahler J: **Quantitative analysis of fission yeast transcriptomes and proteomes in proliferating and quiescent cells**. *Cell* 2012, **151**(3):671-683.
8. Hussain SS, Kayani MA, Amjad M: **Transcription factors as tools to engineer enhanced drought stress tolerance in plants**. *Biotechnology progress* 2011, **27**(2):297-306.
9. Singh K: **Transcription factors in plant defense and stress responses**. *Current opinion in plant biology* 2002, **5**(5):430-436.
10. Udvardi MK, Kakar K, Wandrey M, Montanari O, Murray J, Andriankaja A, Zhang JY, Benedito V, Hofer JM, Chueng F *et al*: **Legume transcription factors: global regulators of plant development and response to the environment**. *Plant physiology* 2007, **144**(2):538-549.
11. Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC: **How rhizobial symbionts invade plants: the Sinorhizobium-Medicago model**. *Nature reviews Microbiology* 2007, **5**(8):619-633.
12. Libault M, Joshi T, Benedito VA, Xu D, Udvardi MK, Stacey G: **Legume transcription factor genes: what makes legumes so special?** *Plant physiology* 2009, **151**(3):991-1001.
13. Carroll BJ, Gresshoff PM: **Nitrate Inhibition of Nodulation and Nitrogen Fixation in White Clover**. *Zeitschrift für Pflanzenphysiologie* 1983, **110**(1):77-88.
14. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L: **Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks**. *Nature protocols* 2012, **7**(3):562-578.
15. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L: **Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation**. *Nat Biotech* 2010, **28**(5):511-515.
16. Guo Y, Cai Z, Gan S: **Transcriptome of Arabidopsis leaf senescence**. *Plant, cell & environment* 2004, **27**(5):521-549.
17. Martin JA, Wang Z: **Next-generation transcriptome assembly**. *Nature reviews Genetics* 2011, **12**(10):671-682.

Chapter 5

An ethylene-NAC transcription factor-Cysteine protease regulatory module triggers senescence in *Medicago truncatula* root nodules

Authors: Vidyadhar Karmarkar*[,1,§], Cezary Smaczniak[1,†], Kerstin Kaufmann[1,†,‡], Marco Busscher[1], Nick Krom[2], Ji He[2], Michael Udvardi[2], Kirankumar S Mysore[2], Jiangqi Wen[2], Erik Limpens[1], Ton Bisseling[1,3] René Geurts*[,1]

Affiliations:

1. Laboratory of Molecular Biology, Department of Plant Science, Wageningen University, Droevendaalsesteeg 1, 6708PB Wageningen, The Netherlands,.
2. The Samuel Roberts Noble Foundation, Ardmore, OK, 73401, USA.
3. College of Science, King Saud University, Post Office Box 2455, Riyadh 11451, Saudi Arabia.

FOOTNOTE:

§ Current Address: Max Planck Institute for Plant Breeding, Carl-von-Linne-Weg 10, 50829 Cologne, Germany.

‡ Current Address: Institute for Biochemistry and Biology, University of Potsdam, Potsdam-Golm, 14476, Germany.

† These authors equally contributed to the work

Abstract

Symbiotic nitrogen fixation by legumes is an environmentally and economically prudent alternative for nitrogen fertilizers. This process occurs in specific organs called the root nodules that host nitrogen-fixing rhizobia. However, symbiotic nitrogen fixation is vulnerable to nodule senescence induced by variable concentrations of soil nitrate. The molecular mechanism regulating nitrate-induced nodule senescence is not well understood. Here, we demonstrate that a *Medicago truncatula* NAC (NAM, ATAF and CUC) transcription factor, *MtNAC920*, is induced after nitrate treatment. *MtNAC920* is an ethylene dependent positive regulator of nodule senescence and directly targets a cysteine protease, *MtCP2*, which was shown earlier to be a marker for nodule senescence. A knockout of the *MtNAC920* can delay premature nodule senescence induced by high concentration of soil nitrate. In summary, herein we elucidate a nitrate-induced nodule senescence module that includes ethylene as secondary messenger and MtNAC920 as the transcriptional switch regulating the cellular senescence program in *M. truncatula*.

Introduction

The global use of nitrogen fertilizers has enabled mankind to significantly enhance food production in the past century, but at the cost of a gamut of environmental problems [1]. To circumvent this problem of anthropogenic agricultural nitrogen, legume based-cropping systems have been traditionally deployed as an environmentally and economically prudent strategy. This utility of legumes to fulfill the nitrogen requirement of agriculture results from their ability of symbiotic nitrogen fixation (SNF) [2, 3]. Notwithstanding the agricultural and environmental benefits of symbiotic nitrogen fixation, breeding for this trait has met limited success. One way to enhance symbiotic nitrogen fixation is by delaying nodule senescence [4], which is triggered at pod-filling, by abiotic stresses and high concentrations of soil nitrogen sources; e.g. nitrate [5, 6]. The nitrate effect on inducing nodule senescence and subsequent termination of symbiosis is known for almost a century (Broun et al., 1932), but the underlying molecular mechanism regulating the onset of senescence is not well understood. A molecular insight in gene regulatory mechanisms controlling nitrate-induced onset of nodule senescence is important for enhancing symbiotic nitrogen fixation through molecular breeding. Here, we have investigated one of the molecular mechanisms triggering nitrate-induced nodule senescence.

Medicago truncatula with its genomic resources [7, 8] is an excellent model system to study nodule senescence. *M. truncatula* forms indeterminate nodules, which can be divided into four developmental zones; meristem, infection zone, nitrogen fixation zone and senescence zone. The persistent apical meristem continuously adds new cells to the nodule in a distal-to-proximal direction. A large fraction of these cells become infected by rhizobia that are present apoplastically in the infection zone. The internalized bacteria are engulfed in a plant-derived membrane and differentiate into so-called bacteroids forming organelle-like structures called the symbiosomes [9]. A single nodule cell can contain hundreds of symbiosomes that encapsulate bacteroids, which express *nif* genes encoding Nitrogenase. This enzyme complex catalyzes the conversion of molecular dinitrogen into ammonia. Symbiosomes can fix molecular dinitrogen for a limited time, generally 4-5 weeks [5, 10]. Subsequently, symbiosome containing cells senesce, resulting in the formation of a so-called senescence zone. In *M. truncatula* nodules senescence occurs in the region most proximal to the root (and most distal from the meristem), and expands over time in a proximo-distal direction [10]. Root nodule senescence coincides with a decline in measurable Nitrogenase activity. Simultaneously, a breakdown of functional leghemoglobin

protein occurs due to the degradation of its heme group, which shifts the nodule color from conical pink into brownish-green [5, 11]. Furthermore, an increased protease activity [5] and at a cellular level, changes in symbiosome integrity are observed. Symbiosome membranes enlarge as they fuse with lytical vacuoles. Symbiosome degradation is a progressive and irreversible response that increases in severity over time [10]. The degradation of symbiosomes can be triggered by various exogenous cues including high concentrations of nitrate [12]. Similar to developmental senescence, nitrate-induced senescence starts at the base of the nodule and progress away towards to nodule tip. These commonalities suggest that, at least in part, a regulatory mechanism is shared between developmental and nitrate-induced senescence.

In plants, biotic or abiotic stress induced senescence is associated with the upregulation of cysteine proteases [13]. Senescence in plants results in massive nutrient remobilization and degeneration of the affected organs. These dramatic effects of plant senescence are conspicuous in aerial and subterranean organs such as leaves, and in case of legumes in rhizobium-induced nitrogen-fixing root nodules. Leaf senescence is a type of programmed cell death triggered by various endogenous and exogenous cues. The various exogenous cues include nutrient stress, drought and temperature whereas internal cues include hormones, reproductive status and age. These cues trigger transcriptional changes that result in increased protease activity and cell death [14]. The transcriptional changes are regulated by various senescence-associated families e.g. NAC, WRKY, HD-ZIP, MYB and AP2-EREBP [15].

NAC (NAM, CUC, ATAF) transcription factors are involved in plant senescence and abiotic stress [16-18]. In *M. truncatula*, *MtNAC969*, was shown to be a negative regulator of nodule senescence [19]. Similarly, in *Arabidopsis thaliana*, senescence-associated cell death is regulated by *AtNAC2* [17], which is also involved in salt-stress response [20]. Induction of *AtNAC2* requires ETHYLENE INSENSITIVE2 (EIN2), a key component of ethylene signaling [21]. Ethylene is known as positive regulator of leaf senescence [14]. Nitrate application also triggers ethylene biosynthesis in legume roots [22], suggesting ethylene as a potential nitrate-induced messenger resulting in nodule senescence. In addition, nodule senescence is accompanied by transcriptional upregulation of genes encoding cysteine proteases [11, 23]. These data suggest nitrate-induced ethylene results in transcriptional reprogramming, possibly via a NAC dependent pathway, required for protease activation and nodule

senescence. Despite this physiological understanding, a study depicting connections between the various molecular nodes has not been described.

In this study, we discovered MtNAC920 as an ethylene dependent positive regulator of nodule senescence. Furthermore, *MtNAC920* directly targets the cysteine protease *MtCP2*, which was shown earlier to be an important molecular marker of nodule senescence. The knockout of functional *MtNAC920* results in delayed nodule senescence and imparts nitrate tolerance marked by significantly reduced induction of *MtCP2* post-nitrate treatment.

Results

MtCP2 triggers nodule senescence

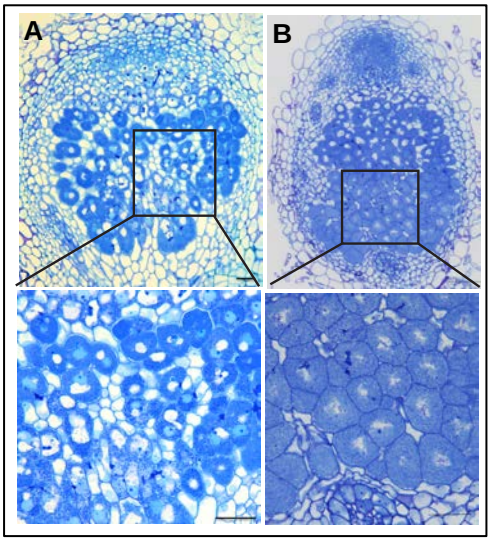

Figure 1. Ectopic expression of *MtCP2*. Toluidine blue stained longitudinal section of a (A) 2 weeks post infection (wpi) nodule expressing *pLB::MtCP2* (n = 20) and (B) 4 wpi control nodules transformed with a empty vector (*pLB:*Empty) (n = 20). Scale bar equals 75 µm.

In *M. truncatula*, six members of the cysteine protease family, named *MtCP1* to *MtCP6*, were proposed to be markers of developmental and dark induced senescence [11]. *In situ* hybridization with a probe against a conserved region of these proteases revealed homogeneity in their expression pattern suggesting a common mechanism regulating their activation [11]. Furthermore, phylogenetic reconstruction revealed that the 6 cysteine proteases represent closely related paralogs [11]. Hence, only *MtCP2* was selected for functional studies. To assess the role of *MtCP2* as an executor of nodule senescence, we ectopically expressed it under the *LB1* leghemoglobin promoter of pea (*Pisum sativum*), which has been used earlier for ectopic expression studies in *M. truncatula* root nodules [24]. Transgenic *M. truncatula* plants harboring the construct *pLB::MtCP2* were generated using *Agrobacterium rhizogenes* mediated transformation. This transformation procedure generates compound plants with non-transgenic shoots and transgenic roots. Plants with transgenic roots were selected based on the presence of DsRED1 derived red fluorescence, and subsequently inoculated with *Sinorhizobium meliloti* to induce nodulation. To study the onset of senescence young root nodules were collected at 2 weeks post infection (2 wpi).

Their phenotype was examined in toluidine blue stained longitudinal sections. We observed premature nodule senescence characterized by symbiosome degradation in *pLB::MtCP2* transgenic plants (n = 20) (Figure 1A). Senescing cells, normally visible in 4-5 weeks old nodules, were visible at the base of the young transgenic root nodules. In contrast, no senescing cells were observed in the fixation zone of young control nodules (Figure 1B). This indicates that ectopic expression of *MtCP2* is sufficient to trigger nodule senescence by potentially targeting the symbiosomes for degradation.

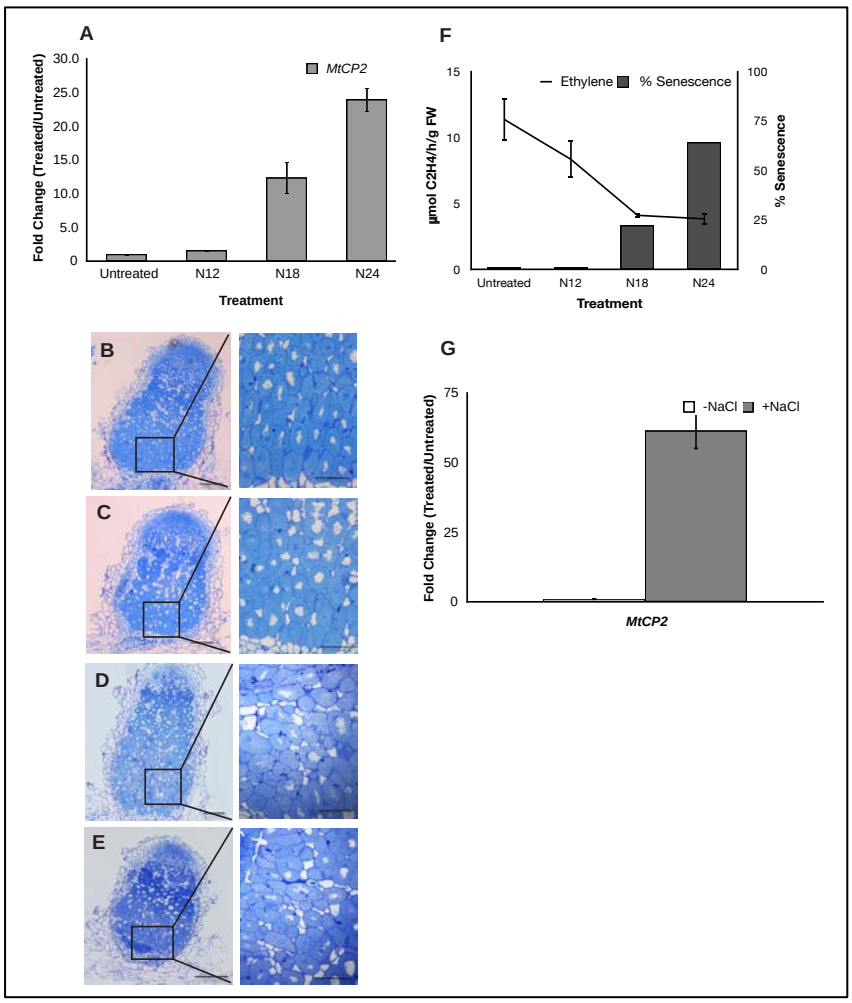

Figure 2. Phenotyping nitrate-induced premature nodule senescence in *M. truncatula.* (A) qRT-PCR expression profile of *MtCP2* in untreated and 12, 18, 24 hrs post application of 15 mM NH_4NO_3 (N12, N18, N24) *pMtCP2* (average of technical triplicates with respective SD). Toluidine blue stained longitudinal sections of 3 wpi nodules (n = 20) of (B) untreated, (C) N12, (D) N18, and (E) N24. Black scale bars indicate 75 µm. (F) Acetylene reduction assay measuring nitrate-induced decline in Nitrogenase activity and quantitation of % senescence at each time point (average of technical triplicates with respective SD). (G) Profiling expression changes of *MtCP2* senescence marker gene (average of technical triplicates with respective SD).

Next, we established a bioassay to activate *MtCP2* expression and trigger nodule senescence. Supplying high concentrations of NaCl [25] or NH_4NO_3 [26] induces nodule senescence in legume root nodules. Hence, we tested the *MtCP2* expression in *M. truncatula* root nodules treated with 15 mM NH_4NO_3 (nitrate). The *MtCP2* expression was monitored at 0 hours (untreated) and 12, 18 and 24 hours after nitrate-treatment (N12, N18 and N24, respectively). At N18, a sharp 12-15x increase in *MtCP2* was observed (Figure 2A), which was accompanied by appearance of senescing cells in 25% of the

nodules (n = 20) (Figure 2B-E). At N24, the *MtCP2* expression increased to 20-25x (Figure 2A) and 75% (n = 20) of the nodules displayed senescing cells (Figure 2B-E). To determine the effect of nodule senescence on nitrogen fixation the Nitrogenase activity was quantified using the acetylene reduction assay [27]. In agreement with the *MtCP2* expression and phenotypic data, the acetylene reduction assay showed a sharp decline in Nitrogenase activity 18 hours after nitrate treatment (Figure 2F). To test whether the activation of *MtCP2* is nitrate-specific, or plays a more generic role in the onset of nodule senescence, its expression was checked in 3 wpi nodules treated with 100 mM NaCl for 24 hours (Figure 2G). NaCl is known as potent inducer of root nodule senescence [25], and our experiment revealed that *MtCP2* was also induced upon salt stress. Taken together this suggests that *MtCP2* is a general executor protease in nodule senescence.

Identifying transcriptional activators of nodule senescence

Transcription factors play an important role in regulating plant senescence and abiotic stress [16-18]. Hence, we aimed to identify transcription factors orchestrating nodule senescence in *M. truncatula*. To obtain a first insight in the transcriptomic changes upon induction of root nodule senescence we performed a single RNA-Seq experiment on the untreated, N12, N18 and N24 nodules. The 100 bp reads generated by RNA-Seq, were used to compute FPKM counts [28]. Because nodule senescence commences ~18 hours after nitrate application, we focused on the differential expression analysis of N18 and N24 nodules. For N18 nodules, we identified 113 putative transcription factors with fold change ($FPKM_{treated}/FPKM_{untreated}$) ≥ 1.8 (Table S1) and 101 putative transcription factors with fold change between 0.1-0.5 (Table S1). Similarly, for N24 nodules we identified 128 putative transcription factors with fold change ≥ 1.8 (Table S1) and 87 TFs with fold change between 0.1-0.5 (Table S1). We identified the top 20 transcription factors that showed maximum fold increase in all 3 treatments. These included, among others, the senescence-associated transcription factor families [15] - HD-ZIP, bHLH, bZIP, AP2-ERF and NAC (Figure 3A, Table S1).

NACs are plant specific transcription factors that play an important role in abiotic stress responses, leaf and nodule senescence [16-19]. Given the similarity between the transcriptomes of senescing *A. thaliana* leaves and *M. truncatula* nodules [10], we hypothesized NACs could also be instrumental in orchestrating nodule senescence. Among the putative NAC transcription factors with differential expression, we identified two NAC transcription factors, Medtr7g085220.1 and Medtr3g096920.2, with ≥ 5x fold increase for

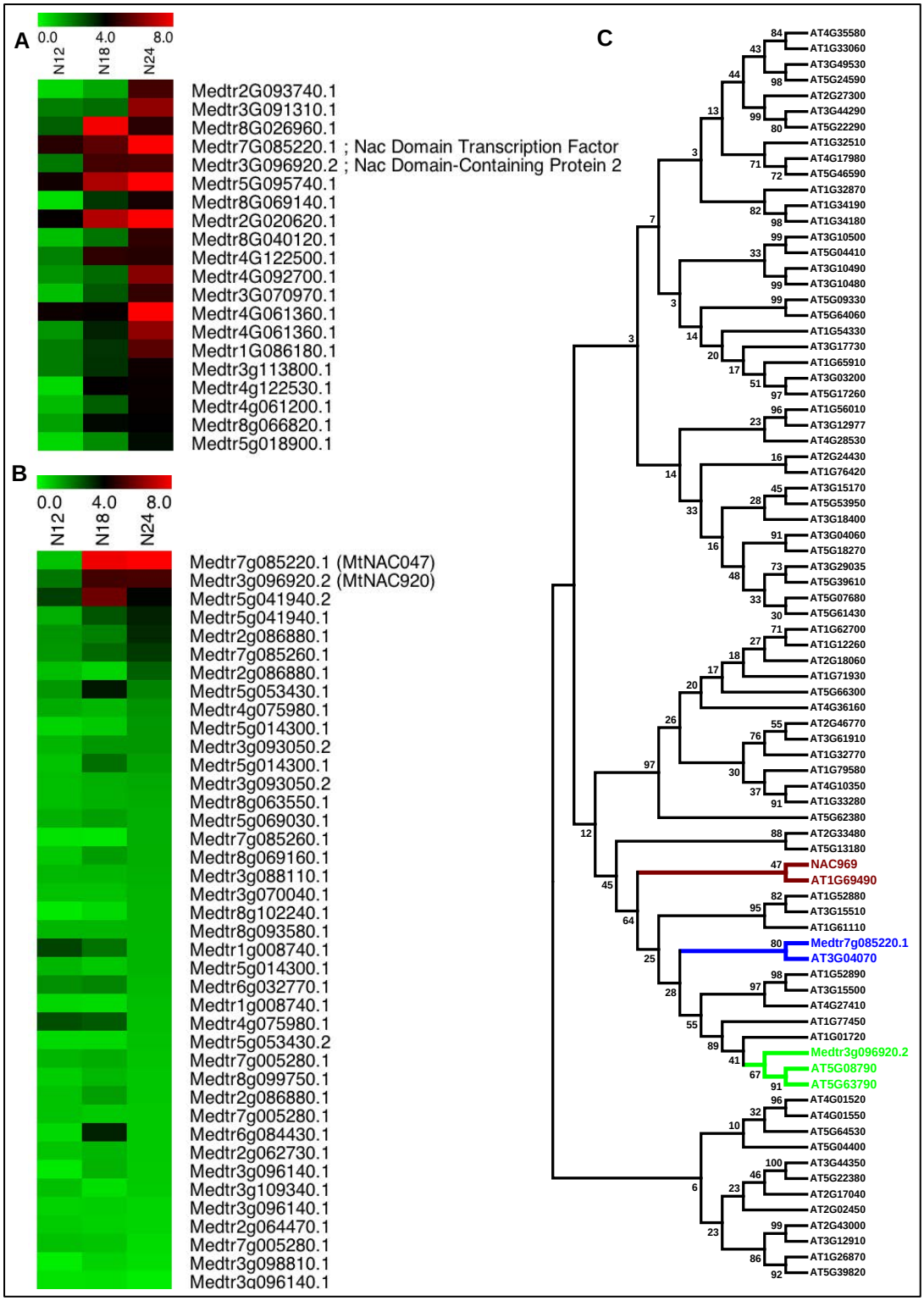

Figure 3. RNA-Seq on nodules of *M. truncatula* plants treated with 15 mM NH_4NO_3. Heatmap depicting fold change (FC) values (FPKMtreated/FPKMuntreated) of (A) top 20 TFs and (B) *MtNAC*s upregulated in the nitrate-treated root nodules 12, 18 and 24 hours post treatment. (C) Topology of an unrooted neighbor- joining phylogeny constructed using the NAC domain sequences of *A. thaliana NAC* genes, Medtr7g085220.1 (MtNAC047), Medtr3g096920.2 (MtNAC920) and MtNAC969. Branch support was obtained from 2,000 bootstrap repetitions. *AtANAC047* and *AtATAF2/AtNAC102* are the closest homologs of Medtr7g085220.1 and Medtr3g096920.2, respectively.

further studies (Figure 3A, B, Table S1). Medtr7g085220.1 and Medtr3g096920.2 are homologous to *A. thaliana AtANAC047* and *AtATAF2/AtNAC102,* respectively (Figure 3C). We refer to Medtr7g085220.1 as *MtNAC047* as it is part of the same orthology group as *AtNAC047*. In case of Medtr3g096920.2, it is difficult to ascertain the precise orthology as it groups together with *AtATAF2* and *AtNAC102* with 91% bootstrap confidence. Therefore we named the M. truncatula gene *MtNAC920,* referring to the last 3 digits of its gene ID.

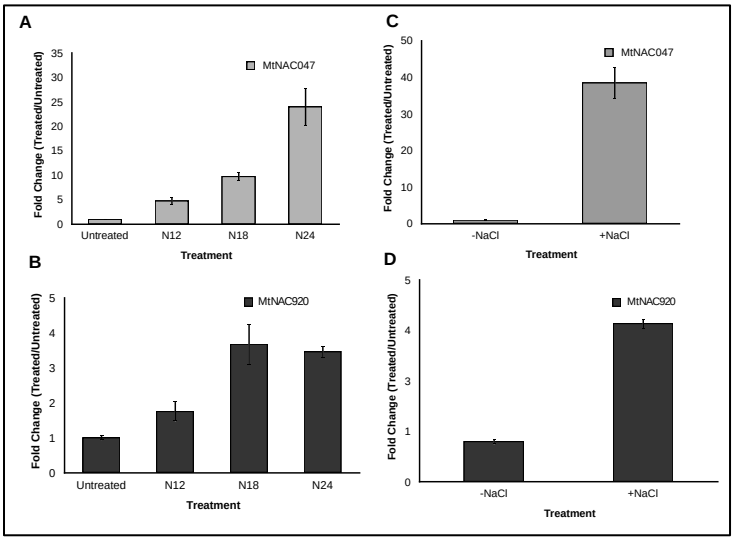

Figure 4. qRT-PCR expression profile of *MtNAC96920* and *MtNAC047* in nodules. (A) *MtNAC047* and (B) *MtNAC96920* expression in untreated and 12, 18, 24 hrs after 15 mM NH_4NO_3 nitrate treatment (N12, N18, N24). (C) *MtNAC047* and (D) *MtNAC96920* expression in untreated and 24 hrs after 100 mM NaCl treatment (average of technical triplicates with respective SD).

To confirm the expression trend of *MtNAC920* and *MtNAC047* that was observed in the RNAseq study, qRT-PCR was conducted on an independent biological replicate of nitrate treated nodules. This study revealed a similar upregulation of both genes in root nodules upon nitrate treatment (Figure 4A, B). Additionally, we found that *MtNAC047* and *MtNAC920* were also upregulated upon salt-stress (Figure 4C, D). This suggests that these NACs could integrate multiple abiotic stress pathways and trigger nodule senescence.

MtNAC920 **triggers nodule senescence by directly activating** ***MtCP2***

To test whether *MtNAC047* and *MtNAC920* have a role in nodule senescence, or a broader role in cell senescence we ectopically expressed both genes using the *A. thaliana Ubiquitin3* (*UBQ3*) promoter [24]. Plants with transgenic roots were inoculated with *S. meliloti* expressing *pnifH::GFP* [29] to induce nodulation. *nifH* encodes a key subunit of the Nitrogenase enzyme complex, thus the GFP signal derived from *pnifH::GFP* activity is a qualitative proxy of the nitrogen fixing status of the nodule. *pUBQ3::MtNAC047* nodules showed a GFP signal (Figure 5) comparable to the *pUBQ3*::Empty (control) nodules

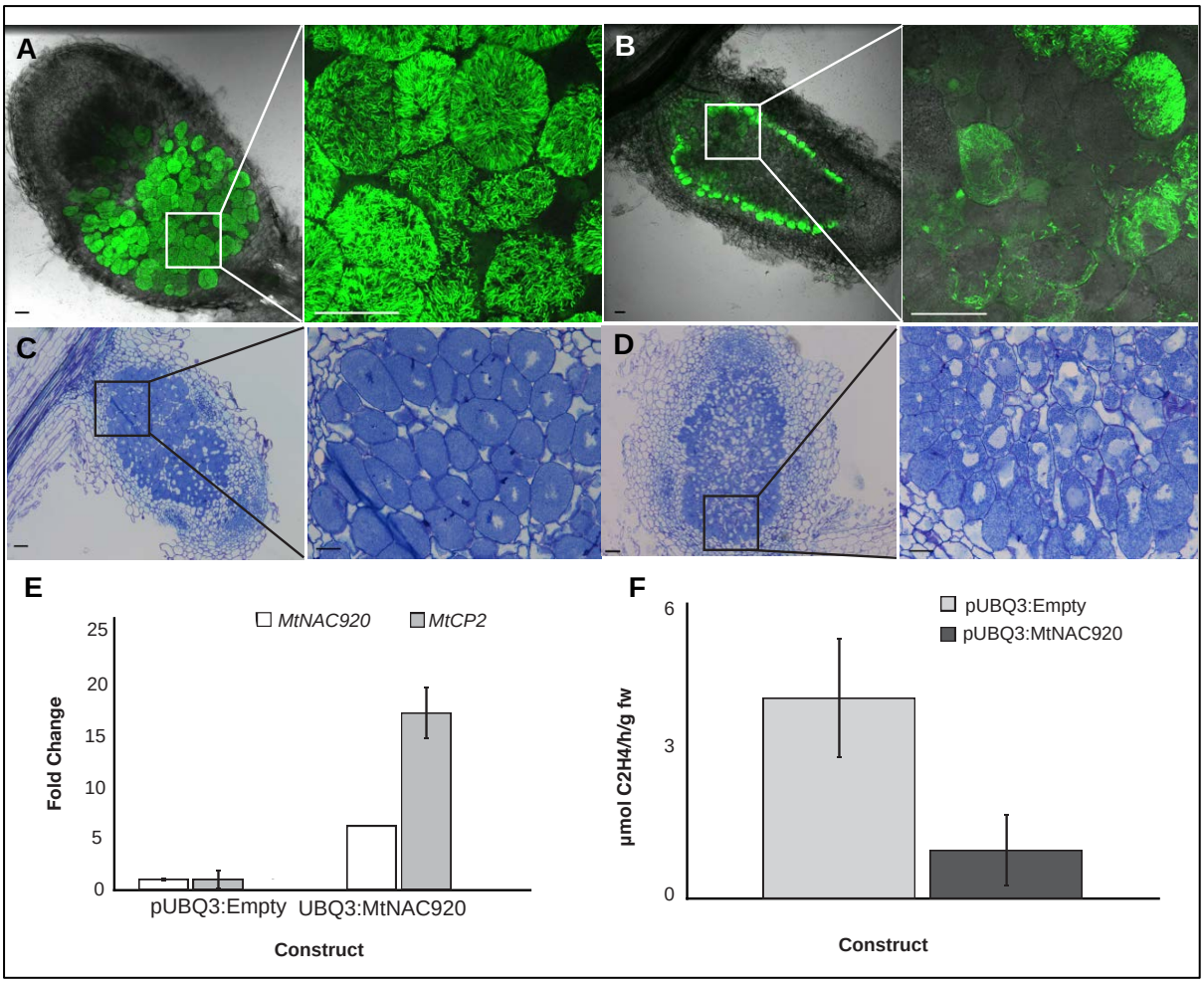

Figure 5. Ectopic expression of *MtNAC920*. *Sinorhizobium meliloti 2011 pnifH::GFP* infected nodules of transgenic *M. truncatula* roots harboring (A) *pUBQ3::*Empty (empty vector) and (B) *pUBQ3::MtNAC920*. Toluidine blue stained longitudinal sections of (C) *pUBQ3::*Empty and (D) *pUBQ3::MtNAC920* nodules (n = 20) 3 wpi. Black scale bars indicate 75 µm. (E) qRT-PCR expression profile of *MtCP2* in transgenic nodules containing *pUBQ3::MtNAC920* compared to *pUBQ3::*Empty control (average of technical triplicates with respective SD). (F) Acetylene reduction assay to compare Nitrogenase activity between *pUBQ3::MtNAC920* and *pUBQ3::*Empty (average of technical triplicates with respective SD).

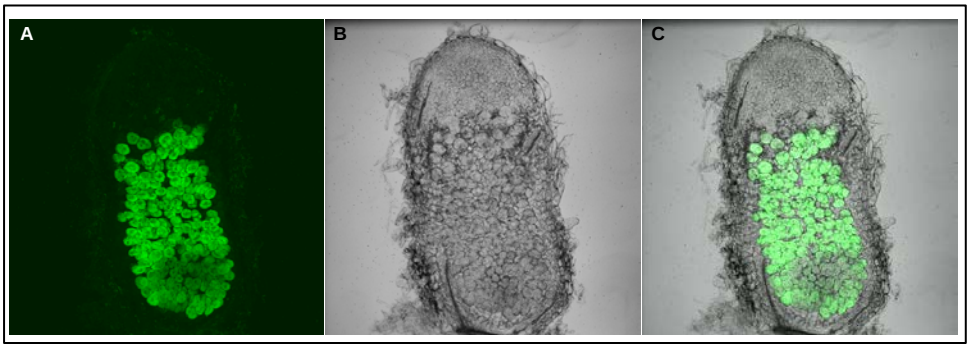

Figure 6. Ectopic expression of *MtNAC047* using pUBQ3:MtNAC047 (n = 20). (A) GFP signal from *S. meliloti* 2011 carrying nifH:GFP in 3 wpi nodules, (B) bright-field image and, (C) overlay of A and B.

(Figure 6). This suggests *MtNAC047* expression is not sufficient to trigger nodule senescence. In contrast, we observed a strong reduction of the GFP signal in the proximal-central region of *pUBQ3::MtNAC920* nodules (Figure 5B). In agreement with the decreased *nifH* expression, the toluidine blue stained longitudinal sections of *pUBQ3::MtNAC920* nodules also showed senescing cells in the proximal-central region (Figure 5D). Senescing cells were not detected in the control nodules (Figure 5C). This suggests that ectopic expression of *MtNAC920* triggers premature nodule senescence. Because senescing nodule cells display bacteroid degeneration and *MtCP2* expression [11], which is also highly upregulated upon nitrate/salt treatment (Figure 2), we compared the expression of *MtCP2* between *pUBQ3::MtNAC920* and control nodules. Ectopic expression of *MtNAC920* triggered an ~18x induction of *MtCP2* as compared to the control nodules (Figure 3E). Furthermore, the senescence in *pUBQ3::MtNAC920* nodules was characterized by measuring Nitrogenase activity with an acetylene reduction assay. In *pUBQ3::MtNAC920* nodules, in agreement with the senescence phenotype, nitrogen fixation declines by 75% as compared to the control nodules (Figure 5F).

To further elucidate the role of *MtNAC920* in *MtCP2* activation, we screened the *M. truncatula Tnt1*-mutant collection and identified a line (NF12171) with a retrotransposon insertion in the last exon of *MtNAC920* (Figure 7A). The homozygous insertion mutant of NF12171 had *MtNAC920* transcript levels < 5% when compared to wild type nodules (Figure 7B). We studied *MtCP2* activation in NF12171 (*Mtnac920*) nodules in presence and absence of nitrate treatment. In the absence of nitrate, *MtCP2* expression in *Mtnac920* was < 5% as compared to wild type nodules. Similarly, after nitrate-treatment, the *MtCP2* expression in *Mtnac920* nodules was only slightly induced (Figure 7C). This demonstrates that MtNAC920 is necessary for *MtCP2* expression and suggests that *Mtnac920* mutant nodules would be more nitrate-tolerant and display delayed nodule senescence. To test this, we first section large nodules that were formed 4 wpi of the *Mtnac920* mutant. In contrast with earlier reports wherein 4-5 weeks old nodules were known to display developmental senescence [5], the *Mtnac920* nodules did not display a senescence zone. Next, such nodules (n = 20) were treated with 15 mM NH_4NO_3 for 24 hrs and were examined for presence of senescing cells. Indeed, alike the untreated mutant nodules (Figure 7D), we did not observe appearance of senescing cells in the toluidine blue stained longitudinal sections of nitrate-treated *Mtnac920*

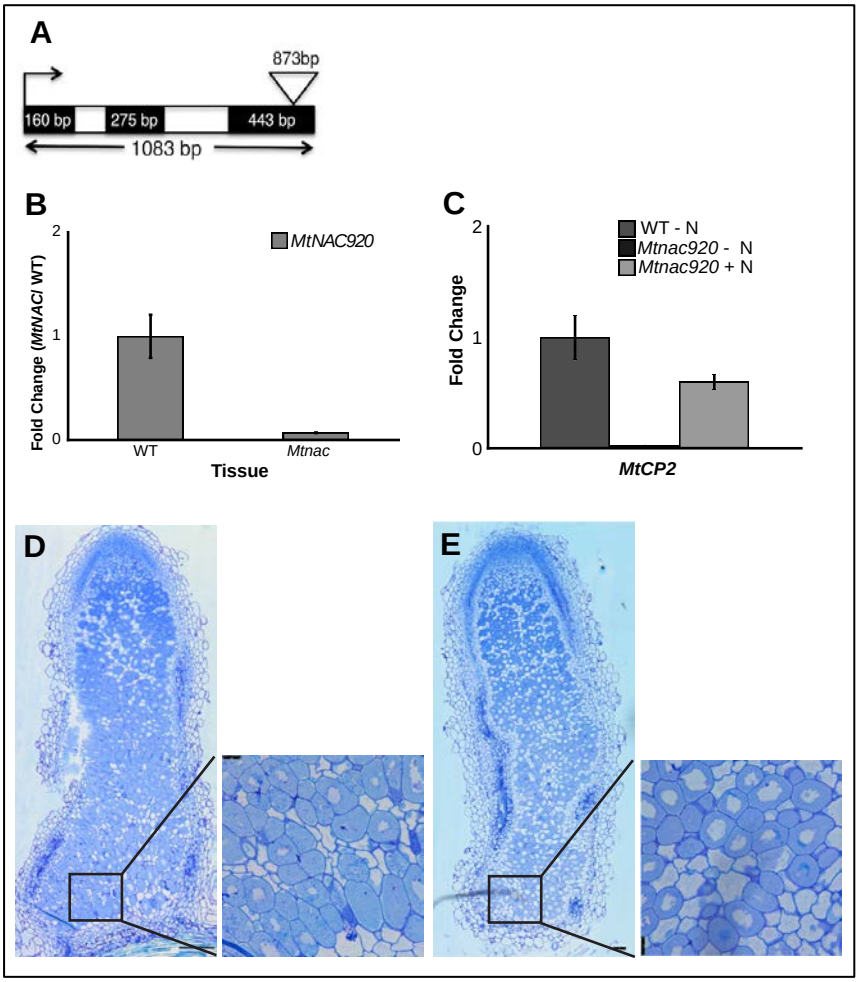

Figure 7. Characterization of *Mtnac* mutant line NF12171. (A) Position of *Tnt1*-insertion in exon 3 of *MtNAC920* (exons indicated as black box). qRT-PCR expression profile of (B) *MtNAC920* in NF12171 compared to WT nodules and (C) senescence marker *MtCP2* in *Mtnac920* ± 15 mM NH_4NO_3 for 24 h (average of technical triplicates with respective SD). Toluidine blue stained longitudinal cross section of a 4 wpi nodule (n = 20) (D) untreated and (E) treated with 15 mM NH_4NO_3 for 24 hours. Black scale bars indicate 75 µm.

nodules (Figure 7E). This indicates that *MtNAC920* is a positive regulator of nodule senescence and *MtCP2* expression.

Because *MtNAC920* and *MtCP2* expression are positively correlated and the close homolog of MtNAC920 in *A. thaliana*, AtATAF2, is a transcriptional activator [30], we hypothesized MtNAC920 directly activates *MtCP2* transcription to trigger nodule senescence. Hence, we performed an *in silico* analysis on the promoters of *MtCP2* (*pMtCP2*) and identified a putative NAC binding site (PNBS) (Figure 8A). The PNBS (ANNNNNTCNNNNNNNCATGT) was defined based on the NAC binding sites described earlier [30, 31]. Two PNBSs were found 299 and 404 bp upstream of the transcriptional start site. We confirmed the physical interaction of MtNAC920 with the 155 bp region containing both the PNBSs within the *MtCP2* promoter using an electrophoretic mobility shift assay (Figure 8B). To confirm the binding of MtNAC920 to this PNBS we conducted a chromatin immunoprecipitation experiment using GFP-tagged MtNAC920 (Figure 8C). Subsequent qPCR on co-presepetated DNA confirmed binding of MtNAC920 on the PNBS promoter region of *MtCP2*

(Figure 8C). Taken together, this suggests that MtNAC920 is a transcriptional switch that directly activates *MtCP2* to trigger symbiosome degradation and nodule senescence.

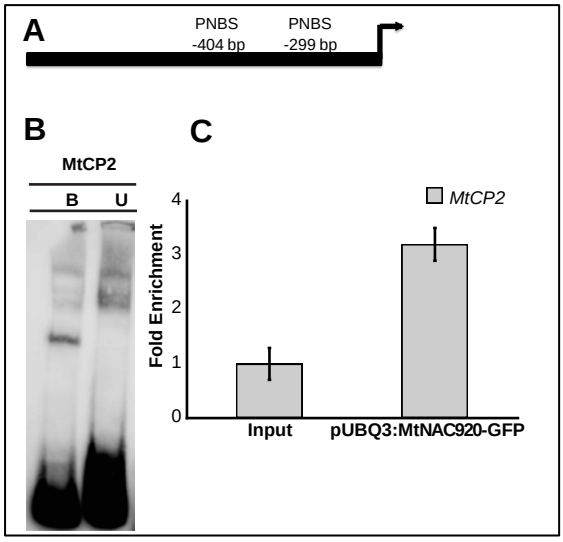

Figure 8. MtNAC920 directly targets *pMtCP2.* (A) Putative NAC binding sites (PNBS) in *MtCP2* promoter (*pMtCP2)*. (B) Electrophoretic mobility shift assay (EMSA) showing *in vitro* binding of MtNAC920 to PNBS within *pMtCP2*. (B = Bound and U = Unbound) (C) Chromatin immunoprecipitation (ChIP) using an anti-GFP antibody followed by qRT-PCR showing *in vivo* binding of MtNAC920 to *pMtCP2* (average of technical triplicates with respective SD).

MtNAC920-MtCP2 activation is dependent on ethylene

Nitrate triggers ethylene biosynthesis in root tissues [32] and ethylene signaling is important in salt response [33]. In *A. thaliana*, the final step of ethylene biosynthesis is regulated by 1-aminocyclopropane-1-carboxylic acid oxidase *AtACO4* (AT1G05010) [34]. To investigate the role of ethylene in the activation of MtNAC920-MtCP2, we studied the expression of the putative *M. truncatula* ortholog of *AtACO4* (named *MtACO4*; Figure 9A). *M. truncatula* plants bearing 3 wpi nodules were treated for 24 hours with 15 mM NH_4NO_3 (nitrate) in presence or absence of 1 µM ethylene biosynthesis inhibitor AVG. Nitrate treatment resulted in a 3x fold induction of *MtACO4*, which was absent in nodules treated with nitrate + AVG as well as untreated nodules (Figure 9B). Similarly, we also observed induction of *MtACO4* in nodules treated with 100 mM NaCl for 24 hours (Figure 9C). This suggests ethylene could act as a secondary messenger in stress-induced activation of MtNAC920-MtCP2.

If ethylene is the secondary messenger in the activation of MtNAC920-MtCP2, then blocking ethylene biosynthesis or signaling should inhibit their stress-dependent activation. We tested this hypothesis in two ways. First, it was tested whether AVG had a negative affect on nitrate induced root nodule senescence. We compared the expression of *MtNAC920* and *MtCP2* in nodules treated with nitrate and nitrate + 1 µM AVG with that of untreated nodules. Unlike nitrate-treated nodules, *MtNAC920* and *MtCP2* expression is not induced in nodules treated with nitrate + 1 µM AVG (Figure 9D, E). This implies that functional ethylene biosynthesis is

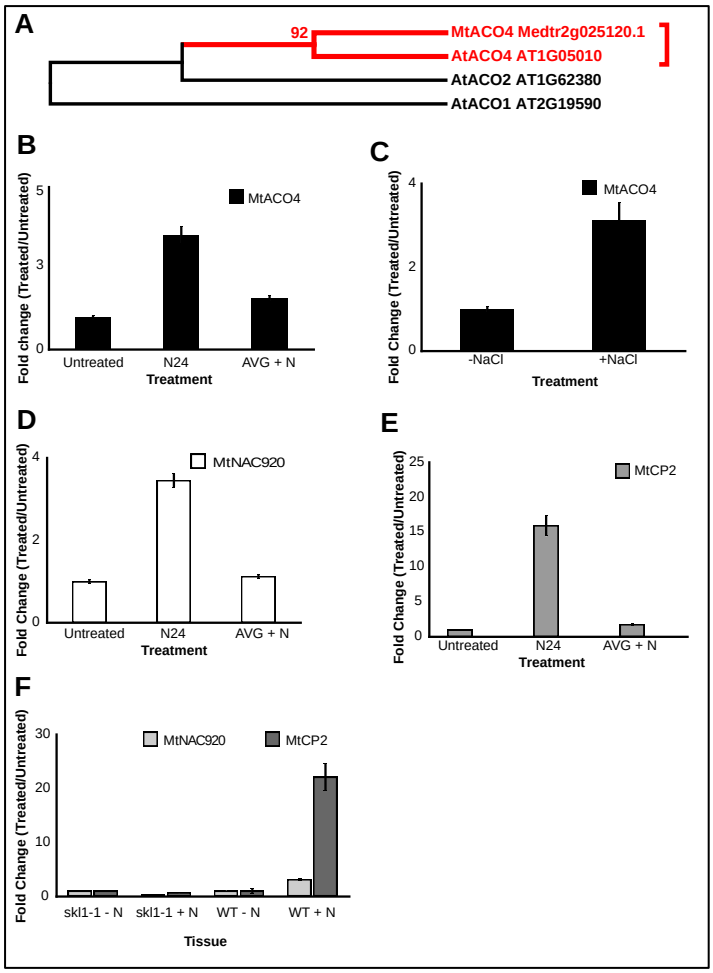

Figure 9. Ethylene is a stress-induced secondary messenger. (A) The *M. truncatula* putative orthologue of *A. thaliana ACO4* (AT1G05010) was identified based on database searches. The orthology group of *MtACO4* and *AtACO4* is shown in an unrooted neighbour-joining phylogeny. Branch support was obtained with 2,000. qRT-PCR expression profiles of (B, C) *MtACO4* expression in root nodules after 24 hrs treatment with 15 mMNH_4NO_3 (N24) or 15 mM NH_4NO_3 + 1 µmAVG (AVG + N) or 100 mM NaCl compared with untreated nodules. (D, E) *MtNAC920* and *MtCP2* expression in N24 and AVG + N treated nodules. (F) *MtNAC920* and *MtCP2* expression in root nodules of *skl1-1* mutant and wild type (WT) ± 15 mM NH_4NO_3 for 24 hrs (average of technical triplicates with respective SD).

necessary for the nitrate-dependent induction of *MtNAC920* and *MtCP2*. To further test the role of ethylene signaling, we used the *M. truncatula ein2*/*skl* mutant that is affected in ethylene signaling (Penmetsa and Cook, 1997; Penmetsa et al., 2008). A *M. truncatula ein2*/*skl* mutant line (*skl1-1*) harboring 3 weeks old nodules was treated for 24 hours with and without 15 mM NH_4NO_3. The expression of *MtNAC920* and *MtCP2* in *skl1-1* in the presence or absence of a nitrate source is comparable to the untreated wild type nodules. In contrast, *MtNAC920* and *MtCP2* expression is highly induced upon NH_4NO_3 treatment (Figure 9F). Taken together, this suggests a functional ethylene signaling network is required for the nitrate-dependent expression of *MtNAC920* and *MtCP2*. The data demonstrate a role of ethylene as a nitrate-induced secondary messenger responsible for induction of *MtNAC920* and *MtCP2*. Thus, *MtCP2* expression and nodule senescence requires an ethylene dependent regulatory module orchestrated by EIN2 and MtNAC920.

Discussion

In this study, we have identified a regulatory module activated upon treating nodulating *M. truncatula* plants with a high concentration of nitrate, which triggers transcriptional upregulation of *MtACO4*, *MtNAC920* and *MtCP2*. Because *MtACO4* is the putative ortholog of *AtACO4*, the key enzyme responsible for ethylene biosynthesis, we used physiological and genetic approaches to attenuate ethylene biosynthesis and/or signaling. Interfering with ethylene biosynthesis by AVG treatment or knocking out ethylene signaling in *ein2/skl* mutant prohibits nitrate-induced upregulation of *MtNAC920* and *MtCP2*. This suggests that ethylene biosynthesis and/or signaling is necessary for transcriptional upregulation of *MtNAC920* and *MtCP2*. Finally, MtNAC920 directly targets *MtCP2*, which upon overexpression, resulted in nodule senescence. Thus, based on our data, we propose a model for nitrate-induced nodule senescence (Fig. 10). In this model, high concentrations of nitrate trigger ethylene biosynthesis. The ethylene signal is transduced further by EIN2/SKL [35], which leads to the upregulation of *MtNAC920*. MtNAC920 directly activates the transcription of *MtCP2* to trigger nodule senescence and symbiosome degradation. Because *MtACO4, MtNAC920* and *MtCP2* upregulation is also observed upon salt treatment, it is likely that stress and nitrate-induced nodule senescence is regulated by a common pathway.

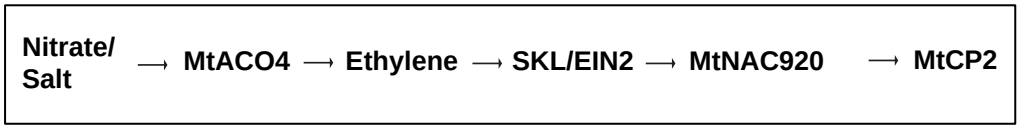

Figure 10. Model for the ethylene-MtNAC920-cysteine protease module regulating premature nodule senescence.

Ethylene signaling plays an important role in nodulation. Chemical inhibition of ethylene biosynthesis leads to increase in persistent rhizobial infections [36]. Conversely, exogenously applied ethylene inhibits nodulation [37] by inhibiting Nod-factor signal transduction [38]. On a similar vein, genetic interference of ethylene signaling pathway in *M. truncatula ein2/skl* mutants results in hypernodulation [35, 39]. Interestingly, ethylene has also been implicated as a secondary messenger in nitrate induced inhibition of nodulation [40]. Our results provide evidence for ethylene signaling in nitrate-induced nodule senescence. Taken together, previous studies and our data suggest ethylene is a key hormone involved in organogenesis and senescence of a nitrogen-fixing root nodule.

The members of the ATAF orthology group of *A. thaliana* are not known to regulate plant senescence. The senescence-associated transcription factors are the members from the

NAP orthology group; e.g. *AtNAP* (AT1G69490) and *AtNAC2* (AT3G15510) are involved in age-induced leaf senescence [17, 41, 42]. In concordance with the role of *NAP*-related genes in senescence, *MtNAC969* that is closely related to *AtNAP* was described to regulate nodule senescence in *M. truncatula* (Figure S1C) [19]. Because *MtNAC969* is a negative regulator of nodule senescence, it will be interesting to study its role in the transcriptional regulation of senescence inducing proteases as well as in nitrate-induced nodule senescence. Nevertheless, our data revealed a novel role for another gene of the ATAF orthology group, *MtNAC920*, in a senescence-related process.

Animal cysteine proteases (named caspases) are constitutively expressed as zymogens and are activated in response to cell-death signals by post-translational mechanisms [43, 44]. However, the stress-induced expression of *MtCP2* in *M. truncatula* root nodules suggests transcriptional control as an important mechanism regulating plant protease activity. This is reminiscent of pathogen dependent induction of the plant vacuolar protease, VPE that is also controlled on a transcriptional level [45, 46]. Herein, we provide insight into a molecular framework for the transcriptional activation of cysteine proteases in legume root nodules. *MtCP2* is closely related to leaf senescence-associated gene *AtSAG12* of *A. thaliana* [11]. AtSAG12 belongs to the family of plant papain-like cysteine proteases (PLCP), which are encoded with an autoinhibitory prodomain that prevents premature activation of the protease [47]. Because *AtSAG12* and *MtCP2* are closely related [11], proteolytic processing of MtCP2 cannot be ruled out. Notwithstanding the possibility of additional post-translational mechanisms of MtCP2 activation, our study provides evidence on transcriptional activation of a cysteine protease in nodule senescence.

We showed that ectopic expression of *MtCP2* triggers premature nodule senescence. This finding is in agreement with a previous study in Chinese milkvetch (*Astragalus sinicus*) wherein silencing of *AsNODF32*, a *MtCP2* ortholog [11], delayed nodule senescence [48]. However, the unsettled issue is how *MtCP2* triggers symbiosome degradation. We hypothesize the vacuolar targeting signal of MtCP2 [11] could target the protease to vacuole-like structures. Because symbiosomes display a vacuolar identity at the onset of senescence [24], these organelles could be the likely targets of degradation by MtCP2. Indeed, a cysteine protease, PsCYP15A, was shown to localize to vacuoles and symbiosomes of pea nodules [49]. Interestingly, PsCYP15A localizes to non-senescing symbiosomes suggesting that proteoloytic processing could be an additional mechanism regulating cysteine protease

mediated nodule senescence. Hence, we hypothesize that a senescence signal triggers transcriptional activation as well as proteolytic processing of the cysteine proteases.

Given the transcriptome and conceptual similarities in leaf and nodule senescence [10, 50], the regulatory module described herein could be important to understand parallels in molecular regulation of senescence in aerial and subterranean organs. However, as compared to leaf senescence, molecular insights in nodule senescence are limited. Hence, in addition to the results described herein, further experiments are necessary to test if ethylene biosynthesis or signaling is sufficient for transcriptional upregulation of *MtNAC920* and *MtCP2*.

From an agricultural perspective, delaying nodule senescence can improve pod-filling in grain legumes [51] and enhance symbiotic nitrogen fixation [4]. However, genetic improvement of this trait has not been successful [4], because of limited understanding of the underlying molecular mechanisms, particularly nodule senescence [5]. The molecular framework identified herein sets the stage to engineer stress-tolerant nitrogen fixation. Targeting transcription factors can be deployed as a strategy to engineer abiotic stress tolerance in plants. NAC transcription factors regulate abiotic stress pathways [18] and have been proposed to be important to engineer abiotic stress tolerance in plants [52]. In the context of environmental degradation resulting from fertilizer-derived anthropogenic nitrogen [53, 54], understanding genetic regulation of nodule senescence is important for breeding legumes with improved nitrogen fixation and reduce our dependence on nitrogen fertilizers.

Experimental Procedures

Plant material

Medicago truncatula accession Jemalong A17 seeds were germinated on Fahraeus medium plates [55]. Five germinated seedlings were transferred to new half-filter Fahraeus medium plates and grown for 1 week in a growing cabinet with 20°C and 16-hr day/8-hr night cycle. Healthy and robust seedlings were then transplanted on perlite and irrigated with Fahreus medium without nitrate. The plants were infected with 2 ml (OD_{600} 0.1) transgenic *Sinorhizobium melilotii* strain 2011 carrying a *nifH*::GFP construct that was constructed according to Starker et al. (2006) [27]. The plants were subsequently irrigated every week with 400 ml water per pot. The growth chamber was set at 20°C and 16-hr day/8-hr night

regime. At 21 dpi, plants were transferred to fresh perlite mixed with 15 mM NH_4NO_3 in liquid Fahraeus medium. The nodules were harvested at 12, 18 and 24 hours (N12, N18 and N24) hours after nitrate treatment. For untreated control nodules, plants were transferred to perlite mixed with liquid Fahraeus medium without nitrate. For genotyping and isolating *Tnt1*-insertion mutants, a reverse PCR-based screening and genotyping was performed as described by Cheng et al., [7]. The primers are listed in table S2.

RNA isolation, cDNA synthesis and qRT-PCR

For RNA-Seq experiments, total RNA was isolated from 21 dpi nodules using the E.Z.N.A. Plant RNA Isolation Kit (Omega Bio-Tek) combined with Qiagen RNase-Free DNase Set for on column DNase treatment. To ensure complete digestion of any contaminating DNA, a second DNase treatment was done using Ambion TURBO™ DNase. RNA quality was evaluated by gel electrophoresis, spectrophotometer and Agilent 2100 Bioanalyzer. RNA was eluted in 20 µl DEPC water. The absence of genomic DNA contamination after DNase treatment was tested using intronic primers designed to amplify a 107 bp intronic fragment of Ubiquitin (TC10247). RNA-seq was conducted by Fasteris (Geneva, Switzerland).

For all qRT-PCR experiments, RNA was isolated as above from 21 dpi nodule tissues followed by a single on-column DNAase digestion. cDNA was synthesized from 1 µg total RNA using Iscript RT-PCR kit (Biorad) in a total volume of 20 µl as instructed in the supplier's manual.

qRT-PCR experiments were conducted in triplicate using 4 ul of the diluted (1:1000) cDNA sample. The qRT-PCR reaction was performed on a MyIQ5 (Biorad) (40 cycles of 95°C for 10 s and 60°C for 1 min). The primers used are listed in table S2.

Acetylene Reduction Assay (ARA)

ARA was performed by modifying the protocol described by Oke et al., (1999) [56]. Briefly, 3 nodulating roots of *M. truncatula* plants were placed in a glass vial and sealed with an air-tight cap. One ml air was removed before injecting 1 ml acetylene. The roots + acetylene was incubated for 1 hour at room temperature. Production of ethylene was analyzed using a gas chromatograph (Chrompack Packard, model 438 with Flame Ionizator Detector)

equipped with a column of Porapak (80/100 mesh). After the assay the nodules were excised to determine fresh weight.

Phylogenetic Analysis

Phylogenetic reconstruction was performed using the neighbour-joining analysis. For NACs this analysis was performed on the basis of the conserved NAC domain sequences of *MtNAC920*, *MtNAC047*, *MtNAC102* and all the *Arabidopsis thaliana* NACs. The NAC protein sequences were downloaded from www.phytozome.net. The protein sequences were aligned using the Clustal W option in MEGA 5.0 by applying Blosum62 matrix, a gap open penalty of 12 and a gap extension penalty of 3. An unrooted phylogenetic tree was reconstructed using neighbor-joining approach and 2,000 bootstrap repetitions to evaluate statistical support of the branches.

Electrophoretic Mobility Shift Assay (EMSA) and Chromatin Immunoprecipitation (ChIP)

MtNAC920 with stop codon was amplified directionally cloned into pENTR TOPO-D (Invitrogen). The PCR primers are presented in table S2. The MtNAC920 entry clone was recombined with the pSPUTK destination vector [57] using the Gateway Technology. EMSA and ChIP experiments were performed as described earlier [57-59].

Constructs and Plant transformation

MtNAC920 and *MtNAC047* open reading frames without stop codons were amplified using the cDNA from 21 dpi nodules. The PCR was performed using Phusion high fidelity Taq polymerase (New England Biolabs) and directionally cloned into pENTR TOPO-D (Invitrogen). PCR primers are presented separately in the table S2. *MtNAC920* and *MtNAC047* were recombined in the Gateway destination vector with *AtUBQ3* promoter created by digesting pK7FWG2 with *HindIII-SpeI* as described by Limpens *et al.,* (2009).

M. truncatula accession Jemalong A17 was used for transformation with *Agrobacterium rhizogenes* strain MSU440 for hairy root transformation using the protocol by Chabaud et al., (2006). *Agrobacterium rhizogenes* mediated root transformation. (http://www.noble.org/MedicagoHandbook/pdf/AgrobacteriumRhizogenes.pdf)

Confocal microscopy

Transgenic roots and nodules were screened for presence of DsRED1 expression using a Leica MZFLIII binocular fitted with HQ470/40, HQ525/50, HQ553/30 and HQ620/60 optical filters. Transgenic nodules were sectioned by hand using a 0.6 mm razor blade and mounted on a glass slide containing a drop of 0.1 M sodium phosphate buffer pH 7.4 + 3% sucrose. The confocal microscopy was performed on Carl-Zeiss Axiovert 100 M equipped with a 543-nm argon and 543-nm helium-neon laser. GFP was excited at 488 nm and detected using a 505-530 nm band pass filter.

RNA-Seq and Transcriptome analysis

We used the TopHat and Cufflinks pipeline at default settings for the RNA-Seq analysis [60]. We used the *M. truncatula* reference genome (Mt. ver. 3.5) and transcriptome (Mt. ver. 3.5.3) as our reference sequence [8]. The unmapped reads were either of low quality, from highly repetitive regions or unsequenced regions of the *M. truncatula* genome, *S. meliloti* sequences, or adapters/chimeric reads and were excluded from further analysis. In the second step, overlapping reads at each locus were grouped together to build a splice graph representing all putative transcript isoforms. Cufflinks at default settings was used to traverse the graph to resolve isoform information.

The transcript and isoform expression was used for digital gene expression (DGE) analysis. We used fragments per kilobase per million reads (FPKM) [61] to estimate to DGE counts. An expressed fragment corresponds to an FPKM value of ≥ 1. Such expressed fragments were classified as unique if it had only 1 best match with the reference sequence and as non-unique if it mapped to 2 or more locations on the reference sequence.

Table S1: RNA-Seq on ammonium nitrate treated root nodules.

Table S2: List of primers used in this study.

References

1. Gruber N, Galloway JN: **An Earth-system perspective of the global nitrogen cycle**. *Nature* 2008, **451**(7176):293-296.
2. Hungria M, Franchini JC, Campo RJ, Graham PH: **The Importance of Nitrogen Fixation to Soybean Cropping in South America**. In: *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment.* Edited by Werner D, Newton W, vol. 4: Springer Netherlands; 2005: 25-42.
3. Drinkwater LE, Wagoner P, Sarrantonio M: **Legume-based cropping systems have reduced carbon and nitrogen losses**. *Nature* 1998, **396**(6708):262-265.
4. Herridge D, Rose I: **Breeding for enhanced nitrogen fixation in crop legumes**. *Field Crops Research* 2000, **65**(2–3):229-248.
5. Puppo A, Groten K, Bastian F, Carzaniga R, Soussi M, Lucas MM, de Felipe MR, Harrison J, Vanacker H, Foyer CH: **Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process**. *The New phytologist* 2005, **165**(3):683-701.
6. Chen PC, Phillips DA: **Induction of Root Nodule Senescence by Combined Nitrogen in Pisum sativum L**. *Plant physiology* 1977, **59**(3):440-442.
7. Cheng X, Wen J, Tadege M, Ratet P, Mysore KS: **Reverse genetics in medicago truncatula using Tnt1 insertion mutants**. *Methods Mol Biol* 2011, **678**:179-190.
8. Young ND, Debelle F, Oldroyd GED, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KFX, Gouzy J, Schoof H *et al*: **The Medicago genome provides insight into the evolution of rhizobial symbioses**. *Nature* 2011, **480**(7378):520-524.
9. Jones KM, Kobayashi H, Davies BW, Taga ME, Walker GC: **How rhizobial symbionts invade plants: the Sinorhizobium-Medicago model**. *Nature reviews Microbiology* 2007, **5**(8):619-633.
10. Van de Velde W, Guerra JC, De Keyser A, De Rycke R, Rombauts S, Maunoury N, Mergaert P, Kondorosi E, Holsters M, Goormachtig S: **Aging in legume symbiosis. A molecular view on nodule senescence in Medicago truncatula**. *Plant physiology* 2006, **141**(2):711-720.
11. Perez Guerra JC, Coussens G, De Keyser A, De Rycke R, De Bodt S, Van De Velde W, Goormachtig S, Holsters M: **Comparison of developmental and stress-induced nodule senescence in Medicago truncatula**. *Plant physiology* 2010, **152**(3):1574-1584.
12. Dart PJ, Mercer FV: **The influence of ammonium nitrate on the fine structure of nodules of medicago tribuloides Desr. and trifolium subterraneum L**. *Archives of Microbiology* 1965, **51**(3):233-257.
13. Pak C, van Doorn WG: **Delay of Iris flower senescence by protease inhibitors**. *The New phytologist* 2005, **165**(2):473-480.
14. Lim PO, Kim HJ, Nam HG: **Leaf senescence**. *Annual review of plant biology* 2007, **58**:115-136.
15. Guo Y, Cai Z, Gan S: **Transcriptome of Arabidopsis leaf senescence**. *Plant, cell & environment* 2004, **27**(5):521-549.
16. Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J: **A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat**. *Science* 2006, **314**(5803):1298-1301.
17. Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG: **Trifurcate Feed-Forward Regulation of Age-Dependent Cell Death Involving miR164 in Arabidopsis**. *Science* 2009, **323**(5917):1053-1057.

18. Nakashima K, Takasaki H, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K: **NAC transcription factors in plant abiotic stress responses**. *Biochimica et biophysica acta* 2012, **1819**(2):97-103.
19. de Zélicourt A, Diet A, Marion J, Laffont C, Ariel F, Moison M, Zahaf O, Crespi M, Gruber V, Frugier F: **Dual involvement of a Medicago truncatula NAC transcription factor in root abiotic stress response and symbiotic nodule senescence**. *The Plant Journal* 2012, **70**(2):220-230.
20. He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY: **AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development**. *The Plant journal : for cell and molecular biology* 2005, **44**(6):903-916.
21. Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR: **EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis**. *Science* 1999, **284**(5423):2148-2152.
22. Ligero F, Lluch C, Olivares J: **Evolution of Ethylene from Roots and Nodulation Rate of Alfalfa (Medicago sativa L.) Plants Inoculated with Rhizobium meliloti as Affected by the Presence of Nitrate**. *Journal of Plant Physiology* 1987, **129**(5):461-467.
23. Naito Y, Fujie M, Usami S, Murooka Y, Yamada T: **The involvement of a cysteine proteinase in the nodule development in Chinese milk vetch infected with Mesorhizobium huakuii subsp. rengei**. *Plant physiology* 2000, **124**(3):1087-1096.
24. Limpens E, Ivanov S, van Esse W, Voets G, Fedorova E, Bisseling T: **Medicago N2-fixing symbiosomes acquire the endocytic identity marker Rab7 but delay the acquisition of vacuolar identity**. *The Plant cell* 2009, **21**(9):2811-2828.
25. Bekki A, Trinchant J-C, Rigaud J: **Nitrogen fixation (C2H2 reduction) by Medicago nodules and bacteroids under sodium chloride stress**. *Physiologia plantarum* 1987, **71**(1):61-67.
26. Streeter J, Wong PP: **Inhibition of legume nodule formation and N2fixation by nitrate**. *Critical Reviews in Plant Sciences* 1988, **7**(1):1-23.
27. Starker CG, Parra-Colmenares AL, Smith L, Mitra RM, Long SR: **Nitrogen fixation mutants of Medicago truncatula fail to support plant and bacterial symbiotic gene expression**. *Plant physiology* 2006, **140**(2):671-680.
28. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L: **Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation**. *Nat Biotech* 2010, **28**(5):511-515.
29. Op den Camp R, Streng A, De Mita S, Cao Q, Polone E, Liu W, Ammiraju JS, Kudrna D, Wing R, Untergasser A *et al*: **LysM-type mycorrhizal receptor recruited for rhizobium symbiosis in nonlegume Parasponia**. *Science* 2011, **331**(6019):909-912.
30. Wang X, Culver J: **DNA binding specificity of ATAF2, a NAC domain transcription factor targeted for degradation by Tobacco mosaic virus**. *BMC plant biology* 2012, **12**(1):157.
31. Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K: **Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter**. *The Plant cell* 2004, **16**(9):2481-2498.
32. Tian QY, Sun P, Zhang WH: **Ethylene is involved in nitrate-dependent root growth and branching in Arabidopsis thaliana**. *The New phytologist* 2009, **184**(4):918-931.

33. Noordally ZB, Ishii K, Atkins KA, Wetherill SJ, Kusakina J, Walton EJ, Kato M, Azuma M, Tanaka K, Hanaoka M *et al*: **Circadian control of chloroplast transcription by a nuclear-encoded timing signal**. *Science* 2013, **339**(6125):1316-1319.
34. Gómez-Lim MA, Valdés-López V, Cruz-Hernandez A, Saucedo-Arias LJ: **Isolation and characterization of a gene involved in ethylene biosynthesis from Arabidopsis thaliana**. *Gene* 1993, **134**(2):217-221.
35. Penmetsa RV, Uribe P, Anderson J, Lichtenzveig J, Gish JC, Nam YW, Engstrom E, Xu K, Sckisel G, Pereira M *et al*: **The Medicago truncatula ortholog of Arabidopsis EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations**. *The Plant journal : for cell and molecular biology* 2008, **55**(4):580-595.
36. Peters NK, Cristestes DK: **Nodule Formation Is Stimulated by the Ethylene Inhibitor Aminoethoxyvinylglycine**. *Plant physiology* 1989, **91**(2):690-693.
37. Lee KH, Larue TA: **Exogenous Ethylene Inhibits Nodulation of Pisum-Sativum L Cv Sparkle**. *Plant physiology* 1992, **100**(4):1759-1763.
38. Oldroyd GED, Engstrom EM, Long SR: **Ethylene Inhibits the Nod Factor Signal Transduction Pathway of Medicago truncatula**. *The Plant Cell Online* 2001, **13**(8):1835-1849.
39. Penmetsa RV, Cook DR: **A Legume Ethylene-Insensitive Mutant Hyperinfected by Its Rhizobial Symbiont**. *Science* 1997, **275**(5299):527-530.
40. Ligero F, Caba JM, Lluch C, Olivares J: **Nitrate Inhibition of Nodulation Can Be Overcome by the Ethylene Inhibitor Aminoethoxyvinylglycine**. *Plant physiology* 1991, **97**(3):1221-1225.
41. Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Carninci P *et al*: **Comprehensive Analysis of NAC Family Genes in Oryza sativa and Arabidopsis thaliana**. *DNA Research* 2003, **10**(6):239-247.
42. Guo Y, Gan S: **AtNAP, a NAC family transcription factor, has an important role in leaf senescence**. *The Plant journal : for cell and molecular biology* 2006, **46**(4):601-612.
43. Sanmartin M, Jaroszewski L, Raikhel NV, Rojo E: **Caspases. Regulating death since the origin of life**. *Plant physiology* 2005, **137**(3):841-847.
44. Riedl SJ, Shi Y: **Molecular mechanisms of caspase regulation during apoptosis**. *Nature reviews Molecular cell biology* 2004, **5**(11):897-907.
45. Rojo E, Martin R, Carter C, Zouhar J, Pan SQ, Plotnikova J, Jin HL, Paneque M, Sanchez-Serrano JJ, Baker B *et al*: **VPE gamma exhibits a caspase-like activity that contributes to defense against pathogens**. *Current Biology* 2004, **14**(21):1897-1906.
46. Hatsugai N, Kuroyanagi M, Yamada K, Meshi T, Tsuda S, Kondo M, Nishimura M, Hara-Nishimura I: **A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death**. *Science* 2004, **305**(5685):855-858.
47. Richau KH, Kaschani F, Verdoes M, Pansuriya TC, Niessen S, Stuber K, Colby T, Overkleeft HS, Bogyo M, Van der Hoorn RAL: **Subclassification and Biochemical Analysis of Plant Papain-Like Cysteine Proteases Displays Subfamily-Specific Characteristics**. *Plant physiology* 2012, **158**(4):1583-1599.
48. Li Y, Zhou L, Li Y, Chen D, Tan X, Lei L, Zhou J: **A nodule-specific plant cysteine proteinase, AsNODF32, is involved in nodule senescence and nitrogen fixation activity of the green manure legume Astragalus sinicus**. *The New phytologist* 2008, **180**(1):185-192.
49. Vincent JL, Brewin NJ: **Immunolocalization of a Cysteine Protease in Vacuoles, Vesicles, and Symbiosomes of Pea Nodule Cells**. *Plant physiology* 2000, **123**(2):521-530.

50. De Michele R, Formentin E, Todesco M, Toppo S, Carimi F, Zottini M, Barizza E, Ferrarini A, Delledonne M, Fontana P *et al*: **Transcriptome analysis of Medicago truncatula leaf senescence: similarities and differences in metabolic and transcriptional regulations as compared with Arabidopsis, nodule senescence and nitric oxide signalling**. *The New phytologist* 2009, **181**(3):563-575.
51. Salon C, Munier-Jolain NG, Duc G, Voisin A-S, Grandgirard D, Larmure A, Emery RJN, Ney B: **Grain legume seed filling in relation to nitrogen acquisition: A review and prospects with particular reference to pea**. *Agronomie* 2001, **21**(6-7):539-552.
52. Tran LS, Nishiyama R, Yamaguchi-Shinozaki K, Shinozaki K: **Potential utilization of NAC transcription factors to enhance abiotic stress tolerance in plants by biotechnological approach**. *GM crops* 2010, **1**(1):32-39.
53. Erisman JW, Sutton MA, Galloway J, Klimont Z, Winiwarter W: **How a century of ammonia synthesis changed the world**. *Nature Geosci* 2008, **1**(10):636-639.
54. Galloway JN, Townsend AR, Erisman JW, Bekunda M, Cai Z, Freney JR, Martinelli LA, Seitzinger SP, Sutton MA: **Transformation of the nitrogen cycle: recent trends, questions, and potential solutions**. *Science* 2008, **320**(5878):889-892.
55. Heidstra R, Geurts R, Franssen H, Spaink HP, van Kammen A, Bisseling T: **Root Hair Deformation Activity of Nodulation Factors and Their Fate on Vicia sativa**. *Plant physiology* 1994, **105**(3):787-797.
56. Oke V, Long SR: **Bacterial genes induced within the nodule during the Rhizobium–legume symbiosis**. *Molecular Microbiology* 1999, **32**(4):837-849.
57. Kaufmann K, Anfang N, Saedler H, Theissen G: **Mutant analysis, protein–protein interactions and subcellular localization of the Arabidopsis Bsister (ABS) protein**. *Molecular Genetics and Genomics* 2005, **274**(2):103-118.
58. Liu W, Kohlen W, Lillo A, Op den Camp R, Ivanov S, Hartog M, Limpens E, Jamil M, Smaczniak C, Kaufmann K *et al*: **Strigolactone biosynthesis in Medicago truncatula and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2**. *The Plant cell* 2011, **23**(10):3853-3865.
59. Kaufmann K, Muino JM, Osteras M, Farinelli L, Krajewski P, Angenent GC: **Chromatin immunoprecipitation (ChIP) of plant transcription factors followed by sequencing (ChIP-SEQ) or hybridization to whole genome arrays (ChIP-CHIP)**. *Nat Protocols* 2010, **5**(3):457-472.
60. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L: **Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks**. *Nature protocols* 2012, **7**(3):562-578.
61. Garber M, Grabherr MG, Guttman M, Trapnell C: **Computational methods for transcriptome annotation and quantification using RNA-seq**. *Nat Meth* 2011, **8**(6):469-477.

Chapter 6

General Discussion

Author: Vidyadhar Karmarkar[1,§]

Affiliations:

1. Laboratory of Molecular Biology, Department of Plant Science, Wageningen

FOOTNOTE:

§ Current Address: Max Planck Institute for Plant Breeding,
Carl-von-Linne-Weg 10, 50829 Cologne, Germany.

Introduction: Senescence – A ubiquitous process

All living organisms are exposed to four inevitable phase changes – birth, disease, aging and death. The process of aging is also called senescence, has been studied at cell, organ and organismal level in all eukaryotes. The eukaryotic cell provides a very good vantage point to classify the types of senescence. All cells go through mitotic and post-mitotic changes, which allow senescence to be classified as – mitotic and post-mitotic [1]. The phase that marks end of the mitotic divisions is called mitotic senescence whereas aging of cells that have ceased mitosis exhibit post-mitotic senescence. Mitotic senescence is observed in yeast and cultured mammalian cells and is also called replicative senescence [2-4]. In contrast, plants exhibit both types of senescence. For instance, mitotic senescence is observed in plant apical meristem, which is consists of rapidly dividing cells that result in new organs such as leaves and flowers. The mitotic arrest in the apical meristem has been referred to as proliferative senescence [5]. In plants, post-mitotic senescence is observed in full-developed organs such as leaves and petals [6, 7]. Herein, I will discuss relevant recent and older literature to gain a bird's eye view of post-mitotic plant senescence (hereafter referred to as plant senescence).

Plant senescence

Plant senescence is an indispensible component integral to their developmental cycle. For instance, senescence is observed in the shrinking of cotyledons of young plants, seasonal change in leaf pigmentation, post-pollination shriveling of petals and senescence of root nodules in legumes [6-8]. The senescence of these organs involves massive remobilization of nutrients that are deposited into developing fruits and seeds. The end result of this nutrient remobilization is often intimately connected with death at cellular, organ or organismal level. The other important changes that are associated with senescence include, degradation of proteins, lipids, nucleic acids and subcellular organelles. The degradation of subcellular organelles varies with the organ e.g. the organelles targeted for disassembly includes chloroplast in leaves, chromoplast in flowers and symbisomes in nodules [6-8]. The organelle degradation is associated with visible symptoms such as leaf yellowing resulting from the degradation of chlorophyll and disassembly of chloroplasts. Likewise, symbiosome degradation in nodules is accompanied by loss of leghemoglobin and increase in bilirubin, which is evident through loss of red color and increase in green color [9]. In spite of these conceptual similarities in senescence of various plant organs, the molecular mechanisms

regulating senescence in each of these organs as well as the commonalities and differences between the mechanisms are not well understood.

Conceptual framework of plant senescence

In order to understand the molecular mechanisms regulating the senescence of plant organs, it is important to put the regulatory nodes into a conceptual framework that is built upon existing literature. To this end, a regulatory framework that laid the conceptual foundation of plant senescence has been proposed earlier [1, 6]. According to the conceptual model, plant senescence could activate a set of genes that may be unique for a pathway or shared between multiple pathways. Each of these pathways could be activated by different exogenous and endogenous cues, which in turn activate a secondary signal that is sensed and/or amplified by respective signal transduction pathways. These secondary signals trigger massive transcriptional changes that activate the senescence-associated genes, which are important for the degradation of organelles, proteins, lipids and nucleic acids, nutrient remobilization and eventually death. Thus, at the heart of this regulatory network are the transcription factors because they interpret the upstream senescence-activating signals and switch on the downstream senescence-associated programs.

Transcriptional regulation of plant senescence

Transcription factors are DNA-binding proteins that bind the *cis*-elements usually located in the 5'-upstream regions of target genes, resulting in their activation or repression. Thus, arguably, transcription factors act as master regulators of various biological processes. Given their central role in regulating plant biological processes, it is not surprising that *Arabidopsis thaliana* has evolved to dedicate 5-6% of its nuclear genome to encode transcription factors [10]. These transcription factors regulate every aspect of plant development, physiology and metabolism. For instance, the plethora of processes regulated by transcription factors include but are not limited to development and patterning [11, 12], seed development [13], plant defense and stress response [14], lignin biosynthesis [15], secondary metabolism [16] and senescence [17, 18].

Among all plant organs, senescence has been well studied in leaves of *A. thaliana*. This is evident from Pubmed search with keywords leaf + senescence, petal + senescence and nodule + senescence, which give 2320, 106 and 120 hits respectively (as of Dec 2013 when

this chapter is being written). Despite the 2320 publications on leaf senescence, only 33 publications (search terms leaf senescence + transcription factors, Dec 2013) describe a functional characterization of transcription factors in the process. This implies the potential role of many transcription factors in leaf senescence is yet to be characterized. Hence in an attempt to identify relevant transcription factors for their role in leaf senescence, transcriptome comparison of senescing and non-senescing leaves has been characterized [17-19]. These studies have primarily used microarrays and qRT-PCR for profiling the transcriptome of senescing and non-senescing leaves. Based on these transcriptome studies, some of the largest families of transcription factors that were shown to be associated with senescence included NAC, WRKY MYB, C2H2 zinc finger and AP2-EREBP. A question that follows is, do these transcription factor families display a time-dependent preponderance in the transcriptome of senescing leaves? Indeed, in one such time-course experiment using microarrays, it was discovered that certain families are overrepresented at different stages of senescence [19]. For instance, members of CCAAT-HAP2 are upregulated in early-mid senescence whereas WRKY genes were upregulated in mid-late senescence. On the other hand, NAC and bZIP are upregulated almost during the entire except very late stages of senescence. Similar striking over-representation was seen for the members of AP2-EREBP genes that were upregulated during mid-stages of senescence [19]. Thus, transcriptome studies have revealed a temporal aspect of the transcriptional control of leaf senescence.

Apart from transcriptome interrogation of senescing leaves in *A. thaliana*, similar studies have been performed on the root nodules of *Medicago truncatula* [20]. In addition, transcriptome comparison of senescing leaves and root nodules of *M. truncatula* was also performed to identify similarities and differences in the transcriptomes of these two aerial and subterranean plant organs [21]. Although both of these studies lacked the rigor for characterization of the temporal changes, they revealed valuable insights to the transcriptome of senescing nodules and their similarities and differences with leaf senescence [21]. Based on the transcriptome of senescing nodules, the transcription factor families that were shown to be over-represented included NAC, bHLH, AP2-ERF, HAP2 and GRAS [20]. Thus, some plant transcription factors, particularly NAC family, is consistently are over-represented in senescing plant organs.

Community resources to characterize plant transcription factors

The three principal approaches for characterizing differentially expressed transcription factors across treatments include microarrays, RNA-Seq and qRT-PCR. Among these approaches, RNA-Seq has emerged as a powerful tool for transcriptome comparison [22]. Despite the power of RNA-Seq, it can still be used as a complement to microarrays [23]. On the other hand, qRT-PCR, although 100 times more sensitive than microarrays [24] but because of its low throughput, is primarily used either for revalidation studies or expression profiling of limited number of genes. However, despite the limitations of throughput in qRT-PCR, it is the best approach if the sole aim of the study is to characterize differentially expressed transcription factors. In addition, the problem of throughput is mitigated because of the community resource platforms created for many plant species including *A. thaliana*, *M. truncatula* and *Lotus japonicus*, *Oryza sativa* [25-28]. These platforms use robotics that increases the throughput, speed and precision of an experiment. Importantly, a qRT-PCR based approach is cost effective, very sensitive, technically less challenging and requires minimum bioinformatics expertise. Thus, given these trade-offs, for a targeted transcriptome comparison studies, qRT-PCR is the best approach.

Systems biology of plant senescence

The power of RNA-Seq or microarrays is not limited to identifying differentially regulated transcription factors. In my opinion, the real power of RNA-Seq and microarrays lies in analyzing the data based on the principle of “guilt-by-association”. According to this principle, the transcriptome data can be leveraged to identify co-regulated modules, *cis*-regulatory elements, predict functions of unknown genes and identify novel alternatively spliced transcripts [29-32]. The high-throughput transcriptome interrogation approaches allow comparison of data across species. For instance, transcriptome data has been used to compare similarities and differences in transcripts between rice and maize [33, 34]. Such cross-species comparision allows discovery of evolutionarily conserved co-expressed genes e.g. conserved and co-expressed genes the plant immunity networks of dicots and monocots were described in *A. thaliana* and *Hordeum vulgare* [35]. Likewise, cross species comparison of transcriptomes identified novel genes involved in leaf development [36].

Apart from transcriptome comparison to identify co-expressed genes, it can also be used to identify *cis*-regulatory elements of co-expressed genes, which increases the sensitivity of the

approach. For instance, using gene ontology analysis identified 13-34% enrichment of co-expressed genes that could be linked functionally. In contrast, the promoter analysis of co-expressed genes identified, 46% genes were enriched for one or more motifs, thus providing a valuable complementary approach to glean relevant information from transcriptome data [30]. This approach was also used in analyzing the promoters of co-expressed genes emerging from a microarray-based transcriptome study of senescing leaves of *A. thaliana*, which revealed the presence of specific abundance of certain *cis*-elements in co-expressed genes [19]. The identification of such senescence responsive *cis*-elements and promoters is important for engineering senescence-tolerance and breeding various crops. For instance, *SAG12*, a senescence-associated gene from *A. thaliana*, was shown to contain several WRKY-binding sites and ABA-response elements [37, 38]. Similarly, *SAG39*, was identified to contain an H-Box and a WRKY binding site [39-41]. The promoters of *SAG12* and *SAG39* were used to target the cytokinin biosynthesis gene *IPT* (*ISO-PENTENYL TRANSFERASE*) from *A. thaliana* and introduced in tobacco and rice resulting in delayed senescence [40, 42].

Given the conceptual and transcriptome similarities in senescing leaves and nodules [21, 43] raises an interesting possibility to compare the transcriptomes of various plant organs to identify conserved regulatory modules. This will allow discovery of fundamental building blocks of life. For such transcriptome comparison studies, *M. truncatula* is arguably the best model because it makes all the plant organs that are known to senesce i.e. leaves, petals, cotyledons and nodules, has a sequenced genome [44], availability of T-DNA lines for reverse genetics [45, 46], community resource for transcriptome studies [27, 47] and increasing amount of transcriptome data [20, 48] that allows possibilities of data mining to design hypothesis-driven experiments. Moreover, because cross-species transcriptome comparison has been successfully implemented [33-36], the information emerging from studies on plant senescence in *M. truncatula* could be available for studies on evolution of plant senescence as well as for translational research to commercial crops.

Senescence and crop productivity

The three fundamental human needs that depend on crops are – food, fiber and fuel. The staple source of food to all higher vertebrates is directly or indirectly derived from grain crops. Likewise, the need for fiber and recently biofuel, also depend on crops. In order to meet these basic needs to the burgeoning world population, it is indispensible to improve crop performance and productivity. The role of senescence in improving crop productivity

and performance was postulated a few decades ago [49]. The premise underlying this postulation was - delaying senescence would be accompanied by maximal photosynthesis and higher yields, which can be measured in increased production of grains, fiber and biomass for fuels. Thus, delayed senescence is an important trait for plant breeding programs.

To this end, many studies have been published demonstrating manipulation of plant senescence can result in improved crop performance and yields in important crops e.g. rice, maize, sorghum, cassava, wheat, barley, soybean and cowpea [reviewed in 50]. The yield of these grain crops can be increased through better grain filling, which depends on the photosynthates derived from vegetative tissues [51]. The pre-anthesis photosynthates contributed to 10-40% of the weight of harvested grains [51]. Pre-mature leaf senescence caused by endogenous or exogenous cues limits the supply of the pre-anthesis photosynthates resulting in reduced yields [52]. Similarly, senescence also influences yield by affecting the post-anthesis remobilization of photosynthates [53, 54]. Another aspect of crop yield is nitrogen, which is derived from the photosynthates [51]. Crop yield is influenced by pre-anthesis nitrogen uptake and post-anthesis nitrogen reallocation [55]. For instance, in barley and wheat up to 90% of nitrogen is reallocated from vegetative tissues [52]. In another example, reduction in the RNA levels of a NAC protein in wheat resulted in ~30% reduction in the protein, zinc and iron content of the grains [56]. Thus, senescence is intimately linked with crop productivity, performance and nutritional content of grains.

Outlook

The world population is expected to reach 9 billion by 2050 indicating global food and nutrition security vis-à-vis climate change are the prime issues facing science and society. Hence, it is imperative to boost the yield and nutritional quality of agricultural produce to feed the world population while causing minimal environmental damage. However, plant senescence adversely affects yield and nutritional quality of agricultural produce. Current efforts in breeding and biotechnology have proven that it is possible to genetically improve crops to manipulate senescence and increase yield and nutrition quality. Thus, delayed senescence should be incorporated as an important trait for gene stacking in plant breeding programs.

References:

1. Gan S: **Mitotic and Postmitotic Senescence in Plants**. *Science of Aging Knowledge Environment* 2003, **2003**(38):re7.
2. Sedivy JM: **Can ends justify the means?: telomeres and the mechanisms of replicative senescence and immortalization in mammalian cells**. *Proceedings of the National Academy of Sciences of the United States of America* 1998, **95**(16):9078-9081.
3. Sedivy JM: **Telomeres limit cancer growth by inducing senescence: long-sought in vivo evidence obtained**. *Cancer cell* 2007, **11**(5):389-391.
4. Takahashi Y, Kuro OM, Ishikawa F: **Aging mechanisms**. *Proceedings of the National Academy of Sciences of the United States of America* 2000, **97**(23):12407-12408.
5. Hensel LL, Nelson MA, Richmond TA, Bleecker AB: **The fate of inflorescence meristems is controlled by developing fruits in Arabidopsis**. *Plant physiology* 1994, **106**(3):863-876.
6. Lim PO, Kim HJ, Nam HG: **Leaf senescence**. *Annual review of plant biology* 2007, **58**:115-136.
7. van Doorn WG, Woltering EJ: **Physiology and molecular biology of petal senescence**. *Journal of experimental botany* 2008, **59**(3):453-480.
8. Puppo A, Groten K, Bastian F, Carzaniga R, Soussi M, Lucas MM, de Felipe MR, Harrison J, Vanacker H, Foyer CH: **Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process**. *The New phytologist* 2005, **165**(3):683-701.
9. Lehtovaara P, Perttila U: **Bile-pigment formation from different leghaemoglobins. Methine-bridge specificity of coupled oxidation**. *The Biochemical journal* 1978, **176**(2):359-364.
10. Riechmann JL, Ratcliffe OJ: **A genomic perspective on plant transcription factors**. *Current opinion in plant biology* 2000, **3**(5):423-434.
11. Moreno-Risueno MA, Van Norman JM, Benfey PN: **Transcriptional switches direct plant organ formation and patterning**. *Current topics in developmental biology* 2012, **98**:229-257.
12. Kaufmann K, Pajoro A, Angenent GC: **Regulation of transcription in plants: mechanisms controlling developmental switches**. *Nature reviews Genetics* 2010, **11**(12):830-842.
13. Sun X, Shantharaj D, Kang X, Ni M: **Transcriptional and hormonal signaling control of Arabidopsis seed development**. *Current opinion in plant biology* 2010, **13**(5):611-620.
14. Singh K: **Transcription factors in plant defense and stress responses**. *Current opinion in plant biology* 2002, **5**(5):430-436.
15. Zhong R, Ye ZH: **Transcriptional regulation of lignin biosynthesis**. *Plant Signal Behav* 2009, **4**(11):1028-1034.
16. Yang CQ, Fang X, Wu XM, Mao YB, Wang LJ, Chen XY: **Transcriptional regulation of plant secondary metabolism**. *Journal of integrative plant biology* 2012, **54**(10):703-712.
17. Guo Y, Cai Z, Gan S: **Transcriptome of Arabidopsis leaf senescence**. *Plant, cell & environment* 2004, **27**(5):521-549.
18. Balazadeh S, Riano-Pachon DM, Mueller-Roeber B: **Transcription factors regulating leaf senescence in Arabidopsis thaliana**. *Plant Biol (Stuttg)* 2008, **10 Suppl 1**:63-75.

19. Breeze E, Harrison E, McHattie S, Hughes L, Hickman R, Hill C, Kiddle S, Kim YS, Penfold CA, Jenkins D *et al*: **High-resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals a distinct chronology of processes and regulation**. *The Plant cell* 2011, **23**(3):873-894.
20. Moreau S, Verdenaud M, Ott T, Letort S, de Billy F, Niebel A, Gouzy J, de Carvalho-Niebel F, Gamas P: **Transcription Reprogramming during Root Nodule Development in *Medicago truncatula***. *PloS one* 2011, **6**(1):e16463.
21. De Michele R, Formentin E, Todesco M, Toppo S, Carimi F, Zottini M, Barizza E, Ferrarini A, Delledonne M, Fontana P *et al*: **Transcriptome analysis of Medicago truncatula leaf senescence: similarities and differences in metabolic and transcriptional regulations as compared with Arabidopsis, nodule senescence and nitric oxide signalling**. *The New phytologist* 2009, **181**(3):563-575.
22. Wang Z, Gerstein M, Snyder M: **RNA-Seq: a revolutionary tool for transcriptomics**. *Nature reviews Genetics* 2009, **10**(1):57-63.
23. Kogenaru S, Yan Q, Guo Y, Wang N: **RNA-seq and microarray complement each other in transcriptome profiling**. *BMC genomics* 2012, **13**(1):629.
24. Horak CE, Snyder M: **Global analysis of gene expression in yeast**. *Functional & integrative genomics* 2002, **2**(4-5):171-180.
25. Czechowski T, Bari RP, Stitt M, Scheible WR, Udvardi MK: **Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes**. *The Plant journal : for cell and molecular biology* 2004, **38**(2):366-379.
26. Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR: **Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis**. *Plant physiology* 2005, **139**(1):5-17.
27. Kakar K, Wandrey M, Czechowski T, Gaertner T, Scheible WR, Stitt M, Torres-Jerez I, Xiao Y, Redman JC, Wu HC *et al*: **A community resource for high-throughput quantitative RT-PCR analysis of transcription factor gene expression in Medicago truncatula**. *Plant methods* 2008, **4**:18.
28. Caldana C, Scheible WR, Mueller-Roeber B, Ruzicic S: **A quantitative RT-PCR platform for high-throughput expression profiling of 2500 rice transcription factors**. *Plant methods* 2007, **3**:7.
29. Movahedi S, Van Bel M, Heyndrickx KS, Vandepoele K: **Comparative co-expression analysis in plant biology**. *Plant, cell & environment* 2012, **35**(10):1787-1798.
30. Vandepoele K, Quimbaya M, Casneuf T, De Veylder L, Van de Peer Y: **Unraveling transcriptional control in Arabidopsis using cis-regulatory elements and coexpression networks**. *Plant physiology* 2009, **150**(2):535-546.
31. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L: **Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation**. *Nat Biotech* 2010, **28**(5):511-515.
32. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B: **Mapping and quantifying mammalian transcriptomes by RNA-Seq**. *Nature methods* 2008, **5**(7):621-628.
33. Bergmann S, Ihmels J, Barkai N: **Similarities and differences in genome-wide expression data of six organisms**. *PLoS biology* 2004, **2**(1):E9.
34. Ficklin SP, Feltus FA: **Gene coexpression network alignment and conservation of gene modules between two grass species: maize and rice**. *Plant physiology* 2011, **156**(3):1244-1256.
35. Humphry M, Bednarek P, Kemmerling B, Koh S, Stein M, Gobel U, Stuber K, Pislewska-Bednarek M, Loraine A, Schulze-Lefert P *et al*: **A regulon conserved in monocot and dicot plants defines a functional module in antifungal plant**

immunity. *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**(50):21896-21901.
36. Street NR, Sjodin A, Bylesjo M, Gustafsson P, Trygg J, Jansson S: **A cross-species transcriptomics approach to identify genes involved in leaf development**. *BMC genomics* 2008, **9**:589.
37. Noh YS, Amasino RM: **Identification of a promoter region responsible for the senescence-specific expression of SAG12**. *Plant molecular biology* 1999, **41**(2):181-194.
38. Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, Schroeder JI: **Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant**. *The Plant cell* 2004, **16**(3):596-615.
39. Lindsay WP, McAlister FM, Zhu Q, He XZ, Droge-Laser W, Hedrick S, Doerner P, Lamb C, Dixon RA: **KAP-2, a protein that binds to the H-box in a bean chalcone synthase promoter, is a novel plant transcription factor with sequence identity to the large subunit of human Ku autoantigen**. *Plant molecular biology* 2002, **49**(5):503-514.
40. Liu L, Zhou Y, Szczerba MW, Li X, Lin Y: **Identification and application of a rice senescence-associated promoter**. *Plant physiology* 2010, **153**(3):1239-1249.
41. Zhang ZL, Xie Z, Zou X, Casaretto J, Ho TH, Shen QJ: **A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells**. *Plant physiology* 2004, **134**(4):1500-1513.
42. Gan S, Amasino RM: **Inhibition of leaf senescence by autoregulated production of cytokinin**. *Science* 1995, **270**(5244):1986-1988.
43. Van de Velde W, Guerra JC, De Keyser A, De Rycke R, Rombauts S, Maunoury N, Mergaert P, Kondorosi E, Holsters M, Goormachtig S: **Aging in legume symbiosis. A molecular view on nodule senescence in Medicago truncatula**. *Plant physiology* 2006, **141**(2):711-720.
44. Young ND, Debelle F, Oldroyd GED, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KFX, Gouzy J, Schoof H *et al*: **The Medicago genome provides insight into the evolution of rhizobial symbioses**. *Nature* 2011, **480**(7378):520-524.
45. Cheng X, Wen J, Tadege M, Ratet P, Mysore KS: **Reverse genetics in medicago truncatula using Tnt1 insertion mutants**. *Methods Mol Biol* 2011, **678**:179-190.
46. Tadege M, Wen J, He J, Tu H, Kwak Y, Eschstruth A, Cayrel A, Endre G, Zhao PX, Chabaud M *et al*: **Large-scale insertional mutagenesis using the Tnt1 retrotransposon in the model legume Medicago truncatula**. *The Plant journal : for cell and molecular biology* 2008, **54**(2):335-347.
47. He J, Benedito VA, Wang M, Murray JD, Zhao PX, Tang Y, Udvardi MK: **The Medicago truncatula gene expression atlas web server**. *BMC bioinformatics* 2009, **10**:441.
48. Boscari A, Del Giudice J, Ferrarini A, Venturini L, Zaffini AL, Delledonne M, Puppo A: **Expression dynamics of the Medicago truncatula transcriptome during the symbiotic interaction with Sinorhizobium meliloti: which role for nitric oxide?** *Plant physiology* 2013, **161**(1):425-439.
49. Thomas H, Stoddart JL: **Leaf Senescence**. *Annu Rev Plant Phys* 1980, **31**:83-111.
50. Gregersen PL, Culetic A, Boschian L, Krupinska K: **Plant senescence and crop productivity**. *Plant molecular biology* 2013, **82**(6):603-622.
51. Yang J, Zhang J: **Grain filling of cereals under soil drying**. *The New phytologist* 2006, **169**(2):223-236.
52. Gregersen PL, Holm PB, Krupinska K: **Leaf senescence and nutrient remobilisation in barley and wheat**. *Plant Biol (Stuttg)* 2008, **10 Suppl 1**:37-49.

53. Himelblau E, Amasino RM: **Nutrients mobilized from leaves of Arabidopsis thaliana during leaf senescence**. *Journal of Plant Physiology* 2001, **158**(10):1317-1323.
54. Thomas H, Howarth CJ: **Five ways to stay green**. *Journal of experimental botany* 2000, **51 Spec No**:329-337.
55. Masclaux-Daubresse C, Reisdorf-Cren M, Orsel M: **Leaf nitrogen remobilisation for plant development and grain filling**. *Plant Biol (Stuttg)* 2008, **10 Suppl 1**:23-36.
56. Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J: **A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat**. *Science* 2006, **314**(5803):1298-1301.

Appendices

Summary

In this thesis, I have researched the topic - how exogenous soil nitrate affects symbiotic nitrogen fixation. Based on my research, I discovered two transcription factors from the NAC (NAM, ATAF, CUC) family, *MtNAC074* and *MtNAC920*, which are upregulated in *M. truncatula* nodules within 24 hours of treatment with 15 mM NH_4NO_3. The nitrate-dependent upregulation of both transcription factors required ethylene biosynthesis and signaling. Chemical interference in ethylene biosynthesis and genetic attenuation of ethylene signaling hindered the nitrate-dependent upregulation of these transcription factors. Importantly, the overexpression of *MtNAC074* and *MtNAC920* affected symbiosome development and nodule senescence respectively. *MtNAC074* directly bound to the promoter region of the members of vesicle associated membrane protein (VAMP) family, *MtVAMP721*. On the other hand, *MtNAC920* directly bound to the promoter region of a cysteine protease, *MtCP2*. *MtVAMP721* genes were shown to be important for exocytotic delivery of cargo important for symbiosome formation [1]. The overexpression of *MtCP2* triggered nodule senescence (this study) whereas the silencing of its homolog delayed nodule senescence in *Astragalus sinicus* [2]. The overexpression of *MtNAC074* and *MtNAC920* led to the downregulation of *MtVAMP721* and *MtCP2* respectively. These data suggested that *MtNAC074* and *MtNAC920* may act as repressors of *MtVAMP721* and *MtCP2* respectively. Based on these data, I propose that *SKL/EIN2* may act as a molecular hub that connects nitrate-derived ethylene signals to the downstream transcriptional response. Through *SKL/EIN2*, nitrate may activate *MtNAC074* or *MtNAC920* to affect nodule development or trigger nodule senescence respectively.

References

1. Ivanov S, Fedorova EE, Limpens E, De Mita S, Genre A, Bonfante P, Bisseling T: **Rhizobium-legume symbiosis shares an exocytotic pathway required for arbuscule formation**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(21):8316-8321.
2. Naito Y, Fujie M, Usami S, Murooka Y, Yamada T: **The involvement of a cysteine proteinase in the nodule development in Chinese milk vetch infected with Mesorhizobium huakuii subsp. rengei**. *Plant physiology* 2000, **124**(3):1087-1096.

Samenvatting

In dit proefschrift heb ik onderzocht hoe exogeen nitraat symbiotische stikstofbinding beïnvloedt. Op basis van mijn onderzoek met de vlinderbloemige *Medicago truncatula*, zijn twee transcriptiefactoren van de NAC familie gevonden, *MtNAC074* en *MtNAC920*. Beiden worden geactiveerd in wortelknolletjes binnen 24 uur na toediening van 15 mM NH_4NO_3. Deze nitraat-afhankelijke regulatie van genexpressie vereist ethyleenbiosynthese en signalering. Het blokkeren van in ethyleenbiosynthese en/of het genetisch muteren van ethyleensignalering interfereert met de nitraat-afhankelijke inductie van deze transcriptiefactoren. Over-expressie van *MtNAC074* of *MtNAC920* leidt tot inactivatie van de symbiose door middel van een proces dat wortelknol-senescentie wordt genoemd. De transcriptiefactor MtNAC074 bindt daarbij aan de promoteren van genen die coderen voor vesikel-geassocieerde membraan eiwitten van de *MtVAMP721* familie, terwijl MtNAC920 bindt aan het promotorgebied van het cysteïne-protease gen *MtCP2. MtVAMP721* genen coderen exocytose-eiwitten die een essentieel zijn voor de opname van de rhizobiumbacteriën in de wortelknolcellen [1]. In mijn onderzoek laat ik zien dat over-expressie van *MtCP2* leidt tot snelle senescentie van de wortelknolletjes, terwijl het uitschakelen van dit gen in de vlinderbloemige *Astragalus sinicus* wortelknol-senescentie vertraagt [2]. In mijn onderzoek toon ik verder aan dat over-expressie van *MtNAC074* en *MtNAC920* leidt tot een verlaagde expressie van respectievelijk *MtVAMP721* genen en het *MtCP2* gen. Deze data suggereren dat MtNAC074 en MtNAC920 functioneren als repressors van respectievelijk *MtVAMP721* en *MtCP2.* Aangezien de regulatie van beide transcriptiefactoren afhankelijk is SKL/EIN2 gecontroleerde ethyleen-signalering concludeer ik dat SKL/EIN2 functioneert als een moleculaire hub tussen exogeen nitraat en regulatie van *MtNAC074* en *MtNAC920* expressie.

Referenties

1. Ivanov S, Fedorova EE, Limpens E, De Mita S, Genre A, Bonfante P, Bisseling T: **Rhizobium-legume symbiosis shares an exocytotic pathway required for arbuscule formation**. *Proceedings of the National Academy of Sciences of the United States of America* 2012, **109**(21):8316-8321.
2. Naito Y, Fujie M, Usami S, Murooka Y, Yamada T: **The involvement of a cysteine proteinase in the nodule development in Chinese milk vetch infected with Mesorhizobium huakuii subsp. rengei**. *Plant physiology* 2000, **124**(3):1087-1096.

Acknowledgment

I take this opportunity to acknowledge the following people, who have greatly supported the work in this thesis.

Prof. dr. Ton Bisseling and Dr. Rene Geurts

Dr. Robin van Velzen, Dr. Cezary Smazcniak, Dr. Kerstin Kaufmann

Dr. Sergey Ivanov, Dr. Stefan Schilderink

Dr. Henk Franssen, Dr. Erik Limpens, Dr. Olga Kulikova, Dr. Elena Federova

Dr. Alessandra Lillo, Dr. Wei Liu

Dr. Michael Udvardi, Nik Krom

Jan Verver, Marijke Hartog, Jan Hontelez

Margo Smit, Danny Hammers, Peter Wesselink, Rik Huismann, Lidija Pavlovic

Olga Zaitseva, Aleksandr Gavrin

Marie-Jose van Lersel, Maria Augustijn

and my family.

Publications

Liu Z. and **Karmarkar V.M.** (2008). Groucho/Tup1 family co-repressors in plant development. *Trends. Plant Science*. 13(3):137-44.

Karmarkar V.M., Kulkarni V.M., Suprasanna P., Bapat V.A., Rao P.S. (2005). Study of radio-sensitivity to Gamma irradiation at different moisture levels in multiple shoot cultures of banana cv. Basrai (AAA). *Physiology and Molecular Biology of Plants*, 11(1):149-152.

Karmarkar V.M., Kulkarni V.M., Bapat V.A., Rao P.S. (2001). Radio-sensitivity of *in vivo* and *in vitro* cultures of banana cv. Basrai (AAA) *Fruits* 56**:** 67-74.

Curriculum Vitae

Vidyadhar Karmarkar was born on 09 September 1976 in Mumbai, India. In 1997, he finished his undergraduate studies in Horticulture from Dr. Balasaheb Sawant Krishi Vidyapeeth. In 1999, he finished his M.Sc. in Agriculture from Dr. Balasaheb Sawant Krishi Vidyapeeth. During his M.Sc., he performed his thesis research on in vitro mutation breeding at Bhabha Atomic Research Center, Mumbai. His work motivated him to develop a career in agriculture biotechnology. To this end, he did a second M.Sc. in Molecular Genetics from Oregon State University, USA. After his M.Sc. from USA, he returned to India to work in the plant breeding and biotechnology sector. After a brief stint in the industry, he started his Ph.D. in Molecular Biology at Wageningen University. At Wageningen, his research on plant-microbe symbiosis shaped his career path in molecular plant microbe interactions. Hence, he did his postdoctoral at the Max Planck Institute for Plant Breeding Research in Cologne, Germany where he worked on molecular connections between the plant circadian clock and immunity. His research experience has resulted in he being offered a job as a lead scientist in Syngenta India, where he will work on vegetable disease resistance.

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: **Vidyadhar M. Karmarkar**
Date: **19 May 2014**
Group: **Molecular Biology, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Nitrate induced nodule senescence	Sep 16, 2010
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, University of Amsterdam	Nov 30, 2012
4th European Retreat of PhD students in Experimental Plant Sciences, Norwich, UK	Aug 15-17, 2012
ExPectationS day (EPS Career Day)	Nov 19, 2012
► **EPS theme symposia**	
EPS Theme 4 symposium 'Genome Plasticity', Radboud University	Dec 11, 2009
EPS Theme 1 symposium 'Developmental Biology of Plants', Wageningen University	Jan 28, 2010
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 22-23, 2013
► **Seminars (series), workshops and symposia**	
Mini-Symposium 'Molecular regulation of seed dormancy'	Oct 20, 2009
Dept Chair Seminars - Ton Bisseling	Sep 08, 2009
CBSG Tech Transfer Symposium	Mar 25-26, 2009
Agrigenomics Seminars on Illumina Sequencing (whole day of seminars)	Mar 03, 2009
► **Seminar plus**	
► **International symposia and congresses**	
21st North American Symbiotic Nitrogen Fixation Conference, USA	Jun 13-18, 2010
9th European Nitrogen Fixation Conference, Switzerland	Sep 06-10, 2010
Next Generation Plant Science Symposium, Max Planck Inst. Plant Breeding, Cologne, Germany	Nov 03-05 Nov 2010
Next Generation Plant Science Symposium, Max Planck Inst. Plant Breeding, Cologne, Germany	Sep 26-28, 2012
Intern. Symosium 'Comunication in Plants and their response to environment', Halle, Germany	May 19-22, 2011
► **Presentations**	
Poster: 21st North American Symbiotic Nitrogen Fixation Conference, USA	Sep 06-10, 2010
Poster: Next Generation Plant Science Symposium, Max Planck Inst. Plant Breeding	Nov 03-05, 2010
Poster: Intern. Symosium 'Comunication in Plants and their response to environment', Halle,	May 19-22, 2011
Poster: ETNA Summer School - Food Security: How can science and policy contribute, Switzerland	Sep 06-14, 2011
International PhD School on Plant Development, Retzbach, Germany	Oct 05-07, 2011
4th European PhD Retreat, Norwich, UK	Aug 15-17, 2012
Poster: Next Generation Plant Science Symposium, Max Planck Inst. Plant Breeding	Sep 26-28, 2012
► **IAB interview**	
Meeting with a member of the International Advisory Board	Oct 29, 2012
► **Excursions**	
Subtotal Scientific Exposure	*17.7 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
ETNA Summer School - Food Security: How can science and policy contribute, Switzerland	Sep 06-14, 2011
International PhD School on Plant Development, Retzbach, Germany	Oct 05-07, 2011
► **Journal club**	
Molecular Biology Journal Club (Organizer)	2009-2010
► **Individual research training**	
Subtotal In-Depth Studies	*6.9 credits**

4) Personal development	*date*
► **Skill training courses (highly recommended)**	
Career Orientation	Feb-Mar 2010
Entrepreneurial Boot Camp, Wageningen University, The Netherlands	Nov 15-20, 2009
Entrepreneurial Boot Camp, University of Wisconsin, Madison, WI, USA	Jul 05-10, 2010
Entrepreneurship Academy, University of California, Davis, USA	Feb 06-09, 2012
► **Organisation of PhD students day, course or conference**	
EPS Expectations Career Day	Nov 19, 2012
► **Membership of Board, Committee or PhD council**	
Member of EPS PhD Council	2010-2011
Subtotal Personal Development	*8.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**34.7**

Herewith the Graduate School declares that the PhD candidate has complied with the educational

** A credit represents a normative study load of 28 hours of study.*

The research described in this thesis was financially supported by the Dutch Science Organization (Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO)) NGI-Horizon (grant 050-71-52).

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Lay-out: Vidyadhar Karmarkar
Printed by: IPSKAMP DRUKKERS