

Mini-Review

Legume small GTPases and their role in the establishment of symbiotic associations with *Rhizobium* spp

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Small GTP-binding genes act as molecular switches regulating myriad of cellular processes including vesicle-mediated intracellular trafficking, signal transduction, cytoskeletal reorganization and cell division in plants and animals. Even though these genes are well conserved both functionally and sequentially across whole Eukaryotae, occasional lineage-specific diversification in some plant species in terms of both functional and expressional characteristics have been reported. Hence, comparative phyletic and correlative functional analyses of legume small GTPases homologs with the genes from other Metazoa and Embryophyta species would be very beneficial for gleaning out the small GTPases that could have specialized in legume-specific processes; e.g., nodulation. The completion of genome sequences of two model legumes, *Medicago truncatula* and *Lotus japonicus* will significantly improve our knowledge about mechanism of biological processes taking place in legume-rhizobia symbiotic associations. Besides, the need for molecular switches coordinating busy cargo-traffic between symbiosis partners would suggest a possible subfunctionalization of small GTPases in Fabaceae for these functions. Therefore, more detailed investigation into the functional characteristics of legume small GTPases would be helpful for the illumination of the events initialized with the perception of bacteria by host, followed by the formation of infection thread and the engulfment of rhizobial bacteria, and end with the senescence of nitrogen-fixing organelles, nodules. In summary, a more thorough functional and evolutionary characterization of small GTPases across the main lineages of Embryophyta is significant for better comprehension of evolutionary history of Plantae, that is because, these genes are associated with multitude of vital biological processes including organogenesis.

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Introduction

Small GTP-binding genes function as signaling molecules regulating vast number of biological processes including early and late secretory pathway,¹⁻³ abiotic and biotic stress signal transduction pathways,⁴⁻⁷ and mitotic spindle assembly^{8,9} in both Metazoa and Plantae. Even though the functional characteristics of these genes have been widely studied in Metazoa species such as human and yeast and in Arabidopsis, there are still many unknowns, especially about Plantae homologs. These genes are generally grouped in four main subfamilies in plants: (1) *ARF/SAR*; (2) *RAB*; (3) *d ROP* (Rho-like proteins in Plants); and (4) *RAN*.¹⁰ Since these genes are essential for cell survival due to their housekeeping functions, they are for the most part strictly conserved with regards to their functional and sequential characteristics across whole eukaryotic kingdom.¹¹⁻¹⁴ However, varying level of functional and expansional divergence has been reported in plants hinting a possible gain of new or related-functions.^{10,15} Thus, with the availability of sequence information from more plant species representing the main branches of Embryophyta, it will be interesting to look into the degree of divergence of these genes in different lineages.

Fabaceous plants are one of few lineages of plants with ability of converting atmospheric nitrogen into usable form by living organisms, ammonia, with specialized organelles in their roots, called nodules.¹⁶ Therefore, understanding of *modus operandi* of nodulation at molecular level is significant. Since the establishment and maintenance of symbiosis organelles involves heavy trafficking of exchange of materials between the symbionts, *Rhizobium* and plant, comparative analyses of functional and phyletic characteristics of Fabaceae small GTPases would further our knowledge about nodulation.

Comparative Analysis of Legume Small GTPases

Even though the complete genome sequence of two model legume plants, *Medicago truncatula* and *Lotus japonicus*, is not available yet, large amount of sequence data including ESTs has accumulated.¹⁷ These two model organisms have been at utmost importance for providing insights about the symbiotic associations between legumes and *Rhizobium* spp. Once the full genome

sequence become available, the comparative analysis between leguminous and non-leguminous genome sequences would be of great value for winnowing the genes evolved to perform Fabaceae-specific biological processes such as nodulation.¹⁸ Since small GTPases are the main switch molecules involving in the regulation of myriad of biological processes such as early and late protein trafficking pathway,¹⁹ high salinity tolerance²⁰ and disease resistance,^{4,21} it is reasonable to assume that these genes could have subfunctionalized in a legume-specific manner to regulate processes related to nodulation. Indeed, we conducted a comparative phyletic from several leguminous species including *Medicago truncatula* and *Lotus japonicus*, and of small GTPases with the homologs from several Metazoa and Plantae species including *Arabidopsis thaliana* and *Homo sapiens* with the purpose of finding out whether a functional, phyletical or expressional divergence has occurred in Fabaceae-lineage of Embryophyta.²² As a result of the study, we identified a total of 10 small GTPases that could have gained nodule-specific spatiotemporal expressional characteristics, implying a possible subfunctionalization. *Medicago* is estimated to have about 120 small GTP-binding genes,²² which is about same number of genes with *Populus trichocarpa*, 117 estimated from a recently published genome sequence^{22,23} and much higher than *Arabidopsis*, 93;¹⁰ which may suggest that these genes could have expanded at differential rates in two main lineages of eudicots, eurosid I and eurosid II. RABs have been the fastest growing subfamily of small GTPases in Fabaceae since the divergence from the most recent common ancestor with *Arabidopsis*. This level of expansion of these genes could also mean that they are likely to gain more lineage-specific functions; e.g., nodule-specific. Even though for the most clades in comparative phyletic tree of Fabaceae RABs are heterogenic; that is, containing orthologs from both legumes and *Arabidopsis*, there are occasional variations in duplication rates and level of sequential divergence between phyletic groups.²² Furthermore, there are RAB cladistic groups that are homogenous for the legume sequences, hinting a likely legume-specific expansion and subfunctionalization. Interestingly, some *Arabidopsis* RAB homologs such as *RABH1a*, *RABH1b*, *RABH1c*, *RABH1d* and *RABH1e* is not associated with any of the leguminous homologs, but are phyletically grouped together with a yeast gene, *YPT6*, and the homologs from poplar, grape and *Chlamydomonas reinhardtii*, and *Physcomitrella patens* RABs (Yuksel et al. unpublished data). This could mean that this group of genes was present in the early common ancestors of both Metazoa and Plantae and later deleted in Fabaceae lineage of Embryophyta. Thus, even though the majority of RABs are conserved across whole Eucaryotae due to their basic roles such as vesicle-mediated trafficking, some of which could have been evolving much faster with regard to both of their functional and sequential characteristics. Even though ROPs (Rho of Plants) are phyletically much closer to each other relative to their Metazoa/Fungi equivalents, RACs, the interfamilial divergence is likely to have been evolving at a higher rate when compared to other three Embryophyta small GTPase subfamilies; *ARF*, *RAB* and *ROP*.^{22,24} For instance, *Arabidopsis ROP6*, did not cluster with any of the genes from other Embryophyta including *Vitis vinifera*,²⁵ *Populus trichocarpa*,²³ *Glycine max*, *Medicago truncatula* and *Lotus japonicus*.

(Yuksel et al. unpublished data). Despite their much recent origin, their regulatory role in shaping the morphology of important plant organelles such as polarized-growth of roots and pollen tubes,^{26,27} and their involvement in plant-pathogen interaction pathways^{5,28} could be some of the factors driving the lineage-specific diversification in these genes. That is because, both of these biological processes; organelle growth and plant-pathogen interactions, are significant for adaptation of plants to new environments. Therefore, the fast pace evolution of ROPs leading to interlineal diversification would be a natural outcome of their functional identities. As for the other major group of small GTPases, ARFs, some phyletic groups such as *Arf1* and *Sar1* are universally conserved in all Eukaryotes with respect to both their functional and sequential characteristics. Nevertheless, there are some clades with the homogenous phyletic characteristics, hinting a possible differential deletion or expansion of some genes following the main speciation events. Thus, more comprehensive analysis of the evolutionary and functional characteristics of small GTPases will be beneficial for providing further insights about the role of these genes in the evolution of Embryophyta. It is quite reasonable to assume that with the increase of the complexity of organisms; more complicated signaling systems will be needed to commensurate with the increased complexity of intra- and intercellular trafficking.

Role of Fabaceae Small GTPases in Nodular Establishment and Maintenance

Nodulation is one of the best known models of symbiotic associations, which evolved over millions of years in 10 different families of Embryophyta, including Fabaceae species such as *Medicago truncatula* and *Lotus japonicus*.¹⁶ These two model legume plants, with an extensive amount of publicly available sequence data, have been the most widely studied organisms for gaining better insights into the molecular components of symbiosis between plants and bacteria. The establishment and maintenance of this highly-specialized, nitrogen-fixing symbiosis organelles (i.e., nodules) involves biologically complex interplay of events between host and symbiont from initial recognition stage and induction of the process,^{29,30} to engulfment of bacteria³¹ and final formation of fully-functioning nodules.³² This intricate teamwork between host, plant and bacteria, *Rhizobium* spp., necessitates the presence of a mechanism regulating the exchange of materials between these partners (Fig. 1). The small GTP-binding proteins are very well-known to be main switch molecules organizing vesicle-mediated trafficking of materials inside cell. Thus, it is logical to think that the main genes playing a major role in the regulation of material transport would likely to be from fabaceous small GTPase homologs. By holding that this statement is true, it could be easily postulated that some members of small GTPases of legumes could be subfunctionalized in the root nodules of legumes as regulatory agents. Furthermore, most of the subfunctionalized genes would be also specialized in terms of their spatiotemporal expressional characteristics. In other words, Fabaceae small GTPases evolved to perform nodule-specific functions are likely to be mainly or solely expressed in nodules. Thus, Yuksel et al.²² attempted to identify small GTPase homologs that are uniquely or preponderantly

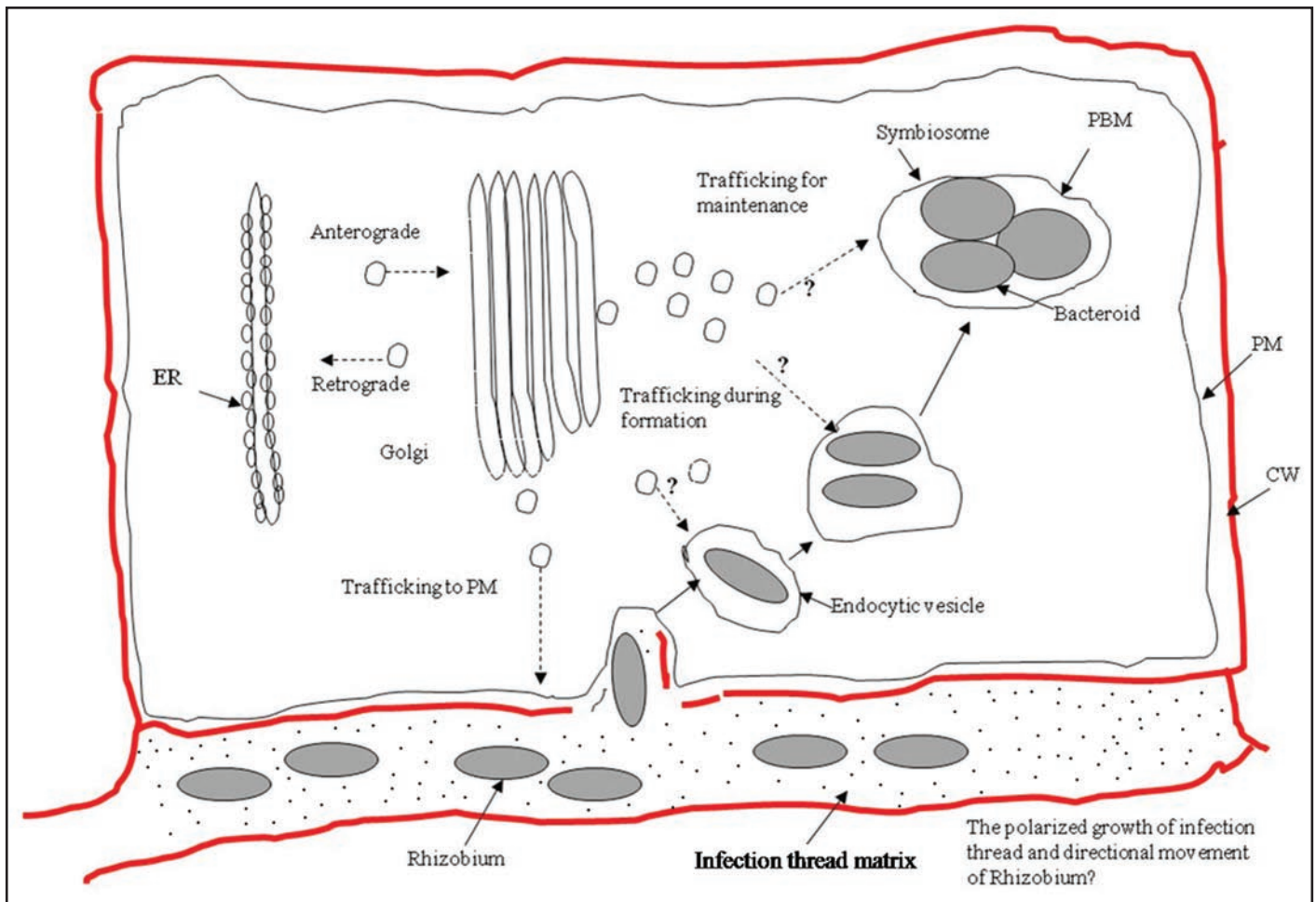


Figure 1. A schematic drawing depicting possible steps where fabaceous small GTPases could play a role during the development and maintenance of nodules. The dashed arrows indicate the possible addresses of the vesicle-coated cargo and the question marks indicate the locations where small GTPases, identified by us, could involve in the regulation of the nodule-specific trafficking. The abbreviations: PM, Plasma Membrane; PBM, Peribacteroid Membrane; CW, Cell Wall; and ER, Endoplasmic Reticulum.

specific to nodules in terms of their spatiotemporal expressional characteristics from two model legume species, *M. truncatula* and *L. japonicus* by comparative analysis of EST sequences obtained from nodular and non-nodular tissues, as a result, 10 small GTPase homologs were fished out as possible candidates directing the shuttling of materials between symbiotic partners. Majority of these homologs belonged to *RAB* subfamily, exactly 7 out of 10. The results obtained from experiments conducted to determine the functional identities of these genes in several plant species is consistent with the hypothesis about the likely switch role of these genes in the regulation of trafficking between symbiotic partners. That is because; some of them are implicated to play a role in vesicle-mediated cargoes to vacuoles.^{33,34} Hence, it is quite reasonable to assume that some members of this subfamily of small GTPases could have evolved to perform nodule-specific functions. Besides, *RAB* proteins are also essential parts of the polarized growth of several plant tissues, e.g., pollen tubes,³⁵ tip growth of root hair cells.³⁶ Therefore, it would not be too farfetched to suggest that some of the Fabaceae *RAB* homologs, identified by us, could be playing a regulatory role in the polarized movement of cell wall

components during the development of specialized symbiosis structures (i.e., infection thread). However, it needs to be made clear that this argument could be easily tested experimentally in one of two model legume species, *Medicago* and *Lotus*, to gain further insights about the involvement of these genes. Furthermore, several previous studies suggested a possible involvement of *RABs* in the establishment and maintenance of nodular structures; e.g., *RAB1p* and *RAB7* in the formation of peribacteroid membrane and rhizobial endocytosis.^{34,35} In conclusion, we believe that *RABs* are likely to be one of the key switch molecules regulating the formation and maintenance of symbiosomes.

Other than 7 legume *RAB* small GTPases, Yuksel et al.²² argued that a *ROP* and two *ARFs* that could be unique to nodules in terms of their spatiotemporal expressional properties. It is rather easy to hypothesize about the putative role of *ROP* protein; that is, this protein could be a significant component of the molecular mechanisms directing polarized growth of the infection thread that requires a complete reorganization of the cytoskeleton³⁷ and the pivotal role of *ROPs* in biological processes requiring an extensive cytoskeletal rearrangement such as pollen tube growth is a well-established

fact.³⁸⁻⁴⁰ As for ARFs, the similar arguments made for RABs with respect to their role in nodules would be true as well. That is because, ARFs from several plant species are implicated in similar roles to RABs such as vacuolar trafficking.⁴¹ In other words, functional overlaps of these two subfamilies of small GTPases in Embryophyta, RABs and ARFs have been observed. Hence, we believe that there could be some members of legume ARFs that could be evolved to perform nodule-specific function.

Conclusions

We suggest that Fabaceae small GTPase homologs could have gained nodule-specific functions including the regulation of cargo-trafficking of materials between symbionts, legume and Rhizobium, and molecular switches coordinating intricate interplay of events that occur during the establishment and maintenance of symbiomes together with tens of already characterized nodular genes. It also needs to be stated that the experimental description of the details of molecular mechanism through which small GTPases take part in the regulation of nodule development and maintenance will greatly contribute to our knowledge about symbiosis biology. Furthermore, better elucidation of the functional and the evolutionary characteristics of small GTPases in whole plant kingdom would be of a great value for furthering our knowledge about how the intracellular trafficking evolved with the increase of organism complexity aftermath of the main divergence points; e.g., the transition from water to land and from nonvascular to vascular systems.

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