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Bacterial Leaf Nodule Symbiosis in Flowering Plants

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Abstract

Bacterial leaf nodule symbiosis within angiosperms is a less known phenomenon compared to the well-documented legume root-*Rhizobium* symbiosis and certainly deserved much more scientific attention. Leaf nodules associated with bacteria was first recognized in *Pavetta* (Rubiaceae) in early twentieth century. Further survey added other members of Rubiaceae, Primulaceae, Dioscoreaceae, and Styracaceae to the short list of plants with specialized bacteria-containing structure in aerial part of plants. The actual role of the bacteria has been questioned by several researchers, mostly due to the problems associated with the identities of these unculturable bacteria. Many progresses have been achieved provided with molecular phylogenetic analysis and also genomic data of the bacteria. Recent evidence from genomic sequences showed the symbiotic bacteria may serve as a defense role in Primulaceae and Rubiaceae, and may increase stress tolerance in Dioscoreaceae. In this article, we reviewed the current knowledge of the bacterial leaf nodule symbiosis in angiosperm. Future research and applications were also discussed.

Keywords: bacterium, convergent evolution, coevolution, endophyte, leaf gland, leaf nodule, symbiosis

1. Introduction

Symbiosis is a long-term and close relationship of two or more biological species that live together for at least part of their life cycle. An endosymbiont is an organism that lives within another, that is, forming endosymbiosis, either intercellular or intracellular [1, 2]. Endosymbionts can be transmitted either vertically (from parent to offspring) or horizontally (from other individuals or environment) [3]. Symbiotic relationships can be obligate or facultative, the former means that one or both symbionts cannot survive without each other. In some cases, the symbiotic relationship provides extra benefits for surviving but is not absolutely necessary to each other, which is known as mutualistic symbiosis.

In plants, various intimate relationships may occur with microbes, which may be friendly, antagonistic, or dynamic [4]. Most of the plant-microbe interaction occurs on surface of the plant body at either underground portion (i.e. rhizosphere) or aerial portion (i.e. phyllosphere), while some interaction occurs within the plant tissues and forms endosymbiosis. The endosymbiotic microbes in plants are also called endophytes, which are often bacteria or fungi. Though the endophytes are ubiquitous in plants, most of these plant-endophyte relationships are not well understood [5]. Most endophytes in plant are without clear function, and only a few are known to be beneficial or harmful to their hosts [6]. In some cases, host plants develop a special structure for harboring the beneficial endophytes. For instance, legumes form root nodule, a specialized structure to house the symbiotic nitrogen-fixing rhizobia. The well-known *Azolla*-cyanobacteria association is another example that the *Azolla* species form a chamber in their leaf, which is often full of nitrogen-fixing cyanobacteria [7, 8]. However, the mutualistic symbiosis with obligate and vertically transmitted is rare in plants. So far, the only two known cases are the *Nostoc*-*Azolla* association [9], and the bacterial leaf nodule or leaf gland symbiosis [10]. The latter will be the main focus of this article.

Bacterial leaf nodule symbiosis, like legume root-*Rhizobium* nodule, consists of a specialized structure of a nodule, or sometimes referred as a “leaf gland”, with bacterial fluid inside a swollen part of the leaves. In angiosperm, the leaf nodules have been discovered in three genera of Myrsinoideae, Primulaceae (*Amblyanthopsis*, *Amblyanthus*, and *Ardisia*), three genera of Rubiaceae (*Pavetta*, *Psychotria*, and *Sericanthe*), *Dioscorea* (Dioscoreaceae), and *Styrax* (Styraceae) (**Figure 1**) [10, 11]. Among these cases, the bacterial leaf nodule symbiosis has been verified microscopically, except in *Amblyanthopsis* and *Amblyanthus* species [10]. In the swollen part of the leaf of *Dioscorea*, the symbiotic bacteria do not invade and digest the plant tissue but are maintaining in the chamber by the host plant, so the term “leaf gland”

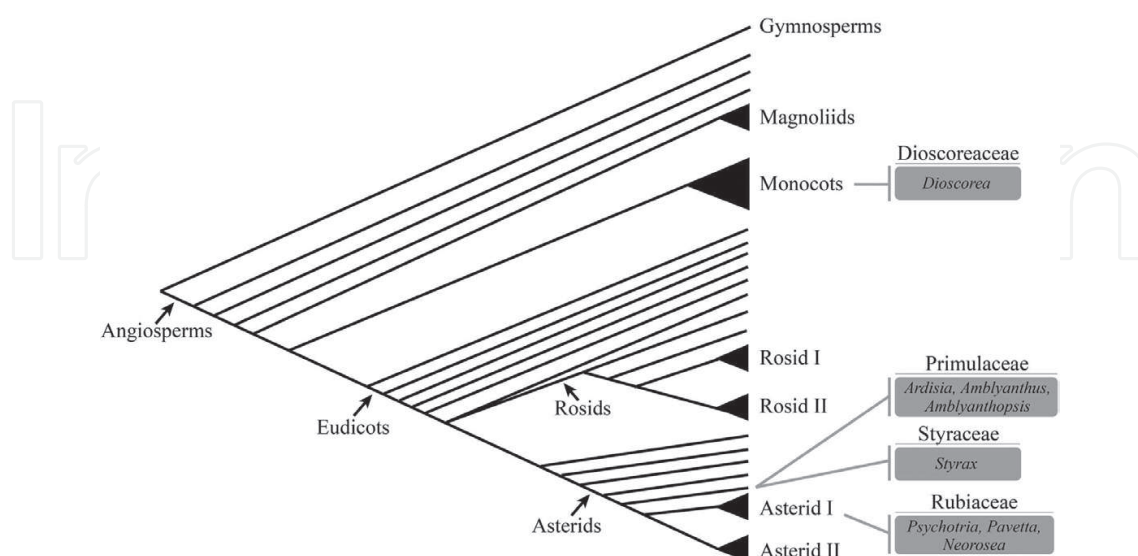


Figure 1. Angiosperm phylogeny modified from APG website [80]. The families and genera with leaf nodulate species are shown next to the corresponding clade.

is used rather than “leaf nodule” in strict sense. In *Styrax*, the bacteria are associating with glandular-trichome-like structure on the shoots and leaves, so the term “leaf gland” is also used in this case.

To date, there are about 530 species reported to have bacterial leaf nodule, which represents about 0.2% of all flowering plants. Nodulated species are mostly distributed through the tropical and subtropical regions in the Old World. The nodulated species of Primulaceae were restricted to tropical and subtropical Asia. The nodulated *Pavetta* has a broader distribution through the tropical and subtropical Africa and Asia. Most nodulated *Psychotria*, *Sericanthe*, and *Dioscorea* are endemic to Africa. In contrast, the recently documented *Styrax camporum* is the only nodulated species that endemic to the New World, Brazil, and South America.

2. The symbionts and the role of the symbionts

2.1. Identity and specificity of the symbionts

The identification of the bacteria of leaf nodule symbiosis is long to be a tempting and controversial question ever since the discovery of these plant-bacteria associations around 1900s [10]. Though many researchers tried to culture and identify the symbionts from the nodulated host plants, many of these isolated bacteria were not congruence with previous morphological observation and were often assigned to different genera (reviewed in [10]). Direct morphological observation of the symbiotic bacteria in all leaf nodulate species is rod-shaped or ovoid- to rod-shaped, Gram-negative, and without flagella [10, 11]. Thus, those isolated/ cultured bacteria might not be the true endophytes in the leaf nodule, but contaminants during cultivation.

In the past two decades, much effort had been made in understanding the true identity of the symbionts and the evolution of the leaf nodule symbiosis relationships, through molecular identification. Based on the 16S rRNA sequences analyses, the bacterial symbionts were successfully identified in many nodulate species, and all belong to Gram-negative beta-proteobacteria. The symbionts of the nodulate species of *Ardisia*, *Pavetta*, *Psychotria*, and *Serricanthe* were identified as *Burkholderia* species, while the symbiont in the *Dioscorea sansibarens* was identified as a novel genus and species, *Orrella dioscoreae* [12–16]. Molecular analyses also demonstrated that the phyllosperic endophyte community of all nodulate species is composed of only one specific bacterial species, which are congruence with the morphological observation. However, the symbionts identity and specificity of the *Styrax camporum* leaf glands remains obscure [11].

2.2. Phylogenetics of the symbionts

It is interesting that the symbionts in nodulate *Ardisia* and nodulated rubiaceaceous plants all belong to the genus *Burkholderia*. *Burkholderia* is an ecologically diverse genus, including both plant and animal pathogens, animal-, plant-, or fungus-associated species, and many free-living species from environment [17]. Phylogenetic studies showed monophyly of the

leaf nodule symbiotic bacteria identified from *Ardisia* species, which suggested a single origin of the leaf nodule symbiosis to *Ardisia* in *Burkholderia* clade [12, 18]. However, the relationships in symbiotic bacteria of nodulated rubiaceous species are much more complicated. The identified symbionts do not form a monophyletic clade corresponding to their host plant genera. The clade consists of symbiotic bacteria from all nodulated rubiaceous hosts that also includes the endophytes identified from non-nodulated *Psychotria* and another non-nodulated rubiaceous genus, *Globulostylis* and some environmental species [14, 15, 19–23] (also see Section 3.3). Other non-nodulated *Burkholderia* species in rubiaceous species together with some plant-associated beneficial and environmental (PBE) species form a monophyletic group. The currently known phylogenetic relationships of *Burkholderia* are showed in **Figure 2**.

The isolated symbiont from the leaf gland of *D. sansibarensis* was assigned to *Orrella dioscoreae*, which belongs to the family Alcaligenaceae in the order Burkholderiales of beta-proteobacteria [16]. The genus name, *Orrella*, is to honor M. Young Orr, who first described the leaf glands of *D. sansibarensis*. Strains isolated from different *D. sansibarensis* populations show limited phylogenetic and phenotypic variation, suggesting the bacteria-plant association in this plant is probably very specific.

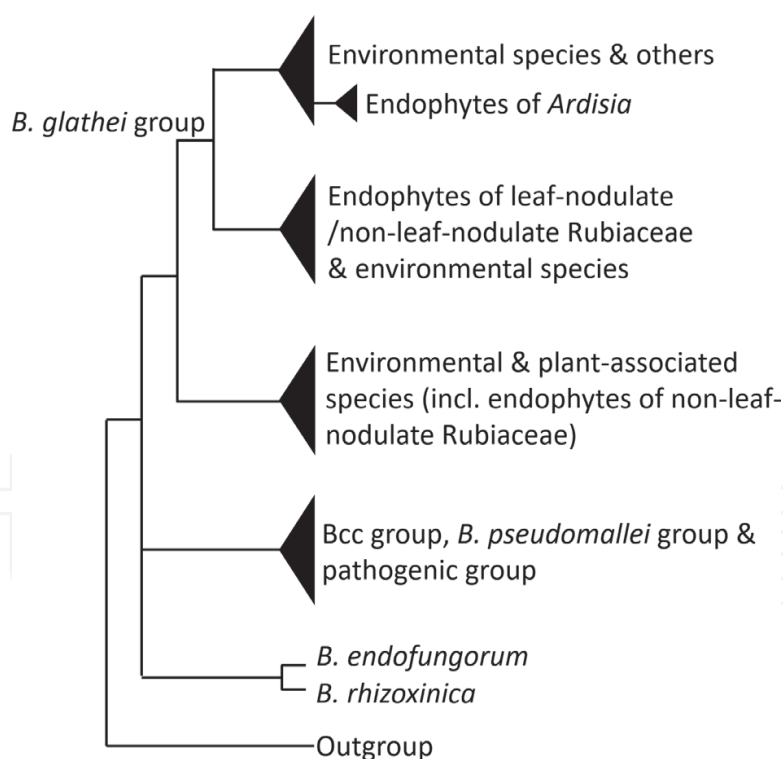


Figure 2. Phylogeny of the bacteria genus *Burkholderia* (modified from [20, 81]). Clades are summarized as triangles. The endophytic bacteria, or symbionts, of nodulate *Ardisia* are a monophyletic group, which embed in a clade consists of mostly environmental species and some symbionts of fungi and insects. The leaf-nodulate *Burkholderia* of the three Rubiaceae genera are not monophyly, respectively, which are mixed together with some endophytes of non-nodulate Rubiaceae, environmental species, and some symbionts of fungi, insects, and plants. Bcc group, *Burkholderia cepacia* complex.

2.3. The role of the symbionts

It has been long speculated for the role of the leaf nodule bacteria and if it is mutualistic. From an evolutionary point of view, it is reasonable to expect a mutualistic relationship in the vertically transmitted symbiosis like the case of the leaf nodule bacteria and their host plants [24]. In a mutualistic symbiosis, the host plants provide a shelter and metabolites for the endophytes. On the other hand, the endophytes may benefit host plants in various form, such as nutrients synthesis, growth regulators synthesis, stress resistance, and defensive metabolites production.

The first proposed function of the leaf nodule or gland symbionts was nitrogen fixation [25, 26], which was widely known from the root nodule association between rhizobia and legumes. However, all in planta studies so far showed negative results on nitrogen fixation, either by using the $^{15}\text{N}_2$ method or acetylene reduction test (reviewed in [10, 27]). Moreover, all authors claimed the leaf nodule endophyte can fix nitrogen based on the isolated bacteria that differs from the ones according to molecular identification. The nitrogen-fixing hypothesis was thus mostly ruled out in *Ardisia*, *Dioscorea*, and rubiaceous plants since the late twentieth century [10]. The lack of nitrogen-fixing-related genes in the symbionts genomic analyses in the currently sequenced genomes also showed disagreement of the hypothesis [19, 28, 29].

In *Ardisia* and *Psychotria*, evidence showed that if the associated bacteria were lost (or decrease to a limit amount, see below), the shoot tip would loss normal function, degenerated to callus (called “cripple” symptom or phenotype), and eventually died within a few years [25, 26, 30–32]. The symptom suggests that the endosymbionts may be responsible for plant normal growth and development, probably by producing hormonal substances. After the hypothesis was proposed, many plant hormones were specified as candidate [10, 25]. Among the various plant hormones, only cytokinin(s), or cytokinin-like substance, was better supported (reviewed in [10, 27]). However, until now there is no direct evidence that the leaf nodulate endosymbionts can produce cytokinin or cytokinin-like substance. In fact, all the evidence supporting the cytokinin-producing hypothesis was obtained by detecting high cytokinin concentration of leaf nodule and nearby tissue, or by the extraordinary need of cytokinin of the plant tissue. Moreover, none of the plant hormone producing genes could be found in all the symbiont genomes sequenced so far [19, 28, 29].

The cripple symptom of leaf nodulate plants were believed to be bacteria free because there are no bacteria in leaf nodule under microscope and no nodule formed on the abnormal leaf [25]. Crippled plants can be grown from seeds which occurred (1) naturally, (2) by either heat treatments, or (3) by antibiotics treatments. The crippled plants were widely used as bacteria-free plants in re-infection experiments and functional analyses. However, the crippled plants were reported to revert to normal state sometimes after a period of time, without additional treatment [27]. Because of the natural recovery of the cripple syndrome, some workers emphasized that crippled plants should not be used for re-infection experiment controls [31, 33]. It is thus reasonable to speculate that the crippled plants are actually bacteria-less rather than bacteria-free. If these plants are not completely bacteria-free, it is interesting to exam the conditions and mechanism how the cripple symptom occur and revert.

Defensive mechanism is another hypothesis for the role of leaf nodule symbiosis to hosts in *Ardisia* and rubiaceous plants. Neal and colleagues first reported the leaf toxicity of *Ardisia crenata* to insect herbivores, whereas the toxicity was not found in another leaf nodule species, *Ardisia crispa* [34]. In contrast to the evidence in *Ardisia*, the chemical defensive hypothesis was better supported in rubiaceous species. A possible linkage between leaf toxicity and endophyte in two non-nodulate genera, *Fadogia* and *Vangueria*, were first revealed in Rubiaceae [35]. Later, the correlation between leaf toxicity and the presence of leaf endophyte was found both in nodulate and some non-nodulate rubiaceous plants [23, 36]. These results suggested that the nodulating endophyte and non-nodulating endophytes may play a similar role on synthesizing defensive chemicals. The defensive role of the symbionts is also supported by the recent genomic analyses (see Section 3.2 for *A. crenata* and Section 4.2 for rubiaceous plants). If the symbionts do serve a defensive role to its host, then it makes sense that removing nodules has no significant effect to next generation seedling growth in *Ardisia* [37]. Nevertheless, the defensive hypothesis has no explanation to the cripple symptom, which was believed causing by losing symbionts. It remains possible that the symbionts in *Ardisia* and *Psychotria* regulate the plant growth and development through unknown regulators, maybe through hormone, or by non-coding RNAs that regulating plant growth-related genes.

In *D. sansibarensis*, the functions of the symbionts were speculated to be beneficial and involved nitrogen fixing at first [38]. Other researchers considered the symbionts as parasites due to the associated bacteria is not always observed in the acumens [39, 40]. However, cultivation experiments showed the host plants grew slowly and looked fragile when the symbiotic bacteria are absent in their leaf glands, while the plants turned vigorous after the bacteria re-infection [32]. This result suggested that the symbiotic bacteria are beneficial to the host and the association is indeed mutualism. Nitrogen fixation in *D. sansibarensis* had not been detected, as in *Ardisia* and rubiaceous plants [10, 32]. However, the genomic analysis suggested that increasing stress tolerance should be the main function of the symbiotic bacteria in *Dioscorea* [16] (see Section 5).

The function of the bacterial symbiosis in *S. camporum* is still unclear. The *S. camporum* extract showed antioxidant and cytotoxic activities, which is a potential source for chemopreventive effect against carcinogenesis [41, 42]. Inspired by other leaf nodule symbiosis, the leaf glandular symbiont in *S. camporum* may also serve a defensive role, although some alternative hypotheses such as nitrogen fixation, plant regulatory hormone synthesis, and stress resistance also cannot be ruled out.

3. Leaf nodule symbiosis in Primulaceae

3.1. The occurrence, initiation, and development

Leaf nodule was found in three woody genera of Primulaceae such as *Ardisia*, *Amblyanthus*, and *Amblyanthopsis* in Myrsinoideae, in which it was formerly recognized as Myrsinaceae. The

genus *Ardisia* contains about 500 species all over the world. Many of them are economically used as ornamental plants and sources of traditional herb medicine. The leaf-nodulated *Ardisia* are classified as subgenus *Crispardisia*, consisting of about 70 species, mostly in the Old World tropical and subtropical regions [43–45]. Both the genus *Amblyanthus* and *Amblyanthopsis* contain four species and are only found in Assam. Two species of *Amblyanthus* and three species of *Amblyanthopsis* have leaf nodules, however, none of them have been examined for bacterial symbiosis. The relationship of the three Myrsinaceae genera is still unclear. The leaf nodules in the three Myrsinaceae genera are ellipsoid or dotted structures that localizing on the margins of the leaves (Figure 3A, C). To be precise, the nodules are on the incisions of the crenation or, less commonly, forming tips of the dentation.

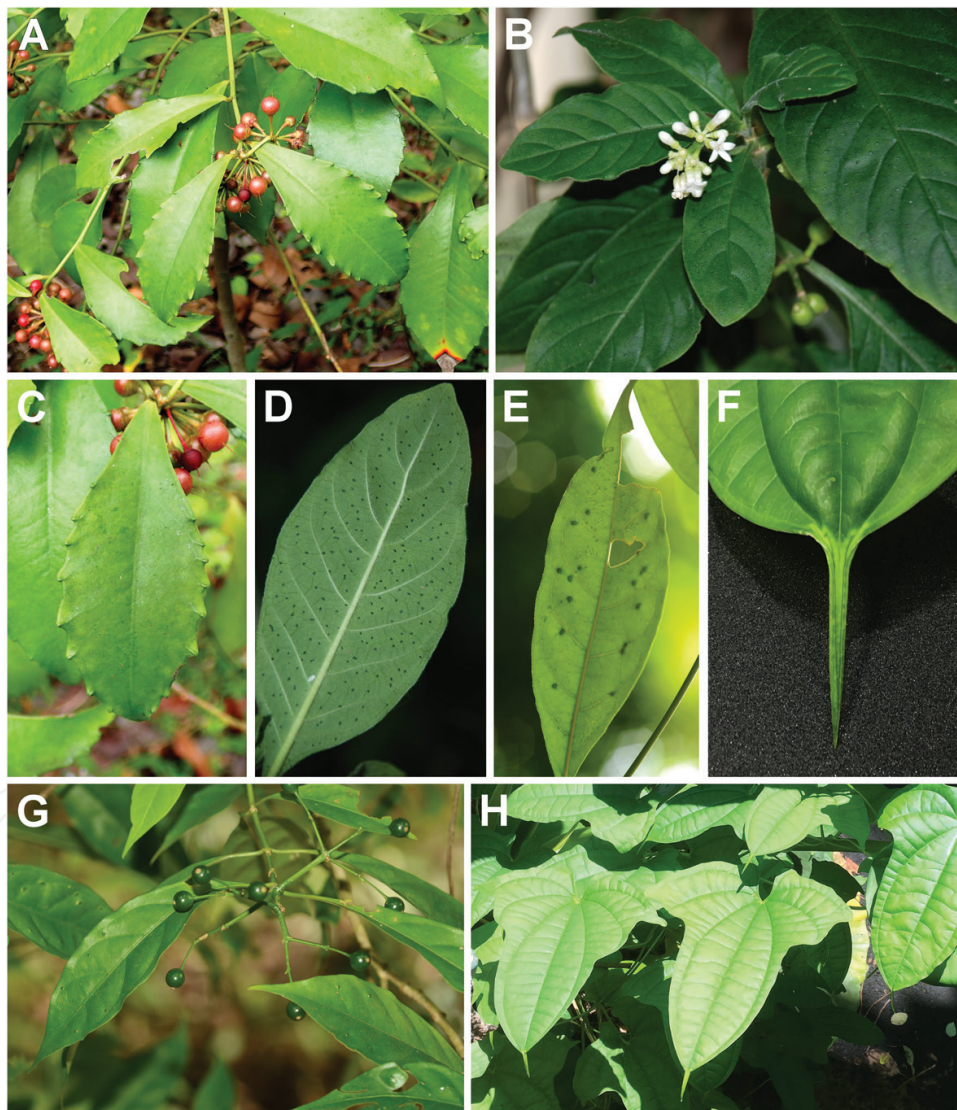


Figure 3. Examples of leaf-nodulated species. (A), (C) *Ardisia cornudentata* Mez (Primulaceae). The leaf nodules are located marginally, forming the tips of the dentation. (B), (D) *Psychotria kirkii* Hiern. (Rubiaceae). The leaf nodules are randomly distributed on the leaf lamina. (E), (G) *Pavetta* sp. (Rubiaceae). The leaf nodules are scarcely distributed on the leaf lamina. (F), (H) *Dioscorea sansibarensis* Pax. (Dioscoreaceae). The leaf apex is swollen and forms a gland.

The most well-known nodulate Myrsinaceae plant is *A. crenata*, or coral berry, which is widely cultivated for ornamental uses. The first description of the bacterial leaf nodule and most of leaf nodule symbiosis studies were demonstrated in *A. crenata* [27]. It is worth noted that *A. crenata* was previously misused as *Ardisia crispa* (Thunb.) A. DC., and most authors in studies before 1990 referred *A. crenata* using the name *A. crispa* [46].

Miehe was the first to describe the swollen structure on leaf margin of *A. crenata* as bacterial nodule [47]. In *Ardisia*, the symbiotic bacteria are observed not only in the leaf nodules but also in the shoot buds [26, 48–50]. The structure and development of the leaf nodules have been described in details [26, 48, 51, 52]. The structure and developmental processes are briefly introduced as below. The shoot bud of *Ardisia* contains a closed chamber forming by two to three tightly convoluted young leaves. The enclosed chamber is full of mucilage that is secreted by the trichomes on both sides of the young leaves. The symbiotic bacteria are harbored within the chamber and supported with the nutrient-rich mucilage. The leaf primordium is immersed in the mucilage until the bud opened. As the leaf initiates and develops, the primordium grows and elongates inward to form a small chamber with some bacterial mucilage. As the leaf matured, some early forming or “precocious” stomata-like pores (or premature hydathode pores referred by some authors) on the leaf margin open and trap some bacterial mucilage to form the nodules in a lysischizogenous manner. At the final stage of nodule maturation, a distinct and sharp boundary of the external vascular sheath and the internal bacterial region can be clearly observed. The surrounding vascular bundle of the nodule indicates that the symbionts could exchange substances with their hosts, and the symbionts probably could produce and translocate certain substances that are beneficial to the host plants. Many of the bacteria in the mature nodule of *A. crenata* were observed to be pleomorphic, as well as in *A. kusukusensis* (**Figure 4**) [18, 47, 52].

In leaf nodulate *Ardisia*, the symbionts were also observed within reproductive tissues and seeds [26, 48, 49]. Based on the distribution of the symbionts on the plant body, the relationship

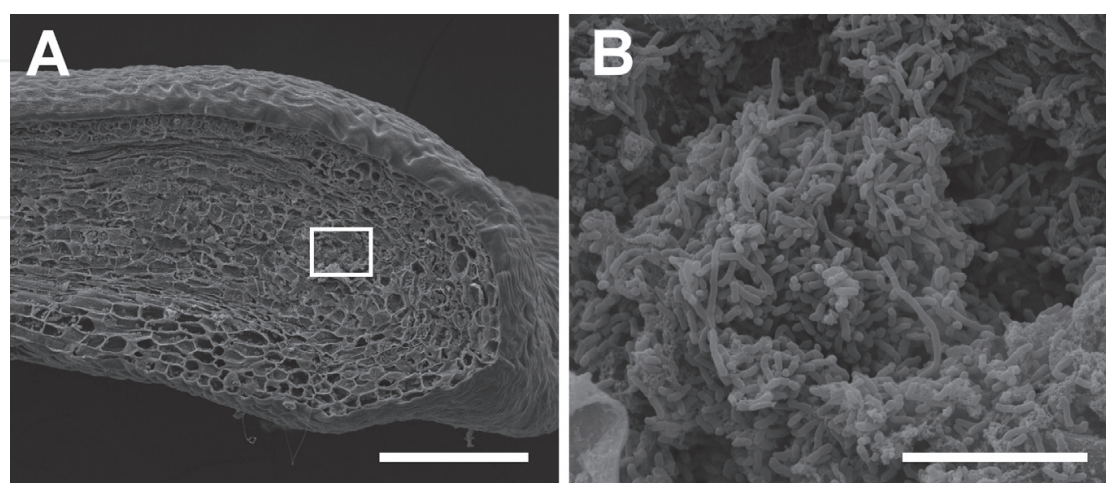


Figure 4. Scanning electron micrographs of a leaf nodule of *Ardisia kusukusensis* Hayata. (A) Cross section of a mature leaf nodule. (B) Rod-shaped endosymbiotic bacteria in the leaf nodule, enlarged from the square region in (A). Bar = 200 μm in (A) and 10 μm in (B). Photos provided by Chuan Ku.

between the symbionts and their *Ardisia* host was speculated to be cyclic, from generations to generations [10]. The inflorescence primordium of *Ardisia* is protected by a small proto-leaf, which is functionally a protective bract. The bract is later rolled-up to form a chamber-like structure just as in the vegetative bud. The inflorescence primordium is immersed in the chamber filling with bacterial mucilage secreted by trichomes on the adaxial surface of the bract. In the early stage of flower development, the calyx develops and forms a new compartment that encloses the rest of flower primordium, and traps some bacterial mucilage inside. The bacterial mucilage then flows into the embryo sac of each ovule and eventually be incorporated into seeds afterwards. The embryo is thus localized in the seed cavity, filling with the bacterial mucilage that is secreted from the trichome on adaxial surface of the cotyledons. When seed begins to germinate, the first true leaf bends backward and roll inward, enclosing the primordium and some bacterial mucilage. Thus, the first shoot bud of the seedling forms and a new life cycle continues.

Interestingly, Gram-negative bacteria were also observed inside the ovary of *Myrsine laetevirens* (also in Primulaceae), a neotropical dioecious tree [53]. The flowers of *M. laetevirens* develop in a similar pattern as in *Ardisia*, and the bacterial mucilage is observed in every stage as pistillate flower development, including the micropyles of ovules. However, the bacteria are absent in staminate flowers, though the mucilage-secreting trichomes is observed. Although the mechanism of bacteria transmission to the embryo sacs is similar with that in *Ardisia*, leaf nodules are absent in *M. laetevirens*. It seems the bacteria are also harbored in the buds in *M. laetevirens*, but it remains unclear whether the bacteria are also present in the leaf or other tissues. In contrast to the non-nodulating rubiaceous plants, the plant-endophyte association of non-nodulating Myrsinaceae plants received much less attention. It is interesting to comprehensively exam whether the associated bacteria are common in non-nodulating Myrsinaceae plants. The identity of the *M. laetevirens* endosymbiotic bacteria and the relationship with other symbiotic bacteria in Myrsinaceae plants are unresolved.

3.2. The origin, phylogeny, and genomics

The *Ardisia* phylogeny showed that the nodulated species form a well-supported monophyletic group, which suggested that the leaf nodule symbiosis only occurred once in *Ardisia*, corresponding to the subgenus *Crispardisia* [12, 18]. Together with the symbionts phylogeny, the origin of the *Ardisia-Burkholderia* association probably evolved only once, both in *Ardisia* and in *Burkholderia*. The estimated origin time of the leaf nodule was about 5 Mya [54]. Cophylogenetic analyses showed weak evidence for *Ardisia-Burkholderia* co-speciation. At least two events of host switching, or horizontal gene transferring, have been postulated based on the comparisons of the bacteria-host phylogenies [12, 18].

The genome of the *Candidatus Burkholderia crenata* (using “*Candidatus*” here referring the bacterium is yet to be cultured, abbreviate to “*Ca.*”) was sequenced recently [29]. The genome size was estimated of 2.85 Mb with one chromosome and two plasmids, based on genome assembling of the next genomic sequencing. However, the result is incongruence with the estimation made by our unpublished data [55]. The estimated genome size and composition

of the *Ca. B. crenata* and *Ca. B. polysticta* by the aforementioned researchers were both around 4.7 Mb with two chromosomes and two large plasmids, based on gel electrophoresis methods. Even if the true genome of *Ca. B. crenata* size is around 4.7 Mb, it is smaller compared to the free-living *Burkholderia*. The reduced genome size and low coding capacity suggest that *Ca. B. crenata* have adapted to a symbiotic life form. The genomic analysis further indicated that *Ca. B. crenata* has lost many essential genes, which should be a result of reductive evolution. Genomic analysis of these bacteria did neither identify nitrogen-fixing-related genes, nor the plant hormone-related genes. However, the incongruent genome size estimation between the assembled genome and gel electrophoresis based estimates suggest the completeness genomic sequences can be improved, and more leaf nodule symbionts genomes of other *Ardisia* would be helpful to draw concrete conclusion.

Nonetheless, two gene clusters related to polyketide and non-ribosomal peptide synthesis were found on the plasmids. The gene clusters have lower GC content and are flanked with transposable elements, suggesting a recent acquisition via horizontal gene transfer. Further studies showed that one of the gene clusters may be correlated with the synthesis of FR900359, a cyclic depsipeptide with potential biomedical application. This result suggested that the symbionts of *A. crenata* may in fact serve a pathogen-defense role for the host.

4. Leaf nodule symbiosis in Rubiaceae

4.1. The occurrence, initiation, and development

The bacterial leaf nodule occurs in three genera of Rubiaceae, that is, *Psychotria*, *Pavetta*, and *Sericanthe*. These three genera belong to different tribes that have no close phylogenetic affinity within Rubiaceae [56]. *Psychotria* belongs to subfamily Rubioideae, while *Pavetta* and *Sericanthe* belong to different tribes of subfamily Dialypetalanthoideae. The shape and distribution of the nodules on the leaves are divergent among genera and species. In general, the leaf nodules of *Psychotria* and *Pavetta* are punctate to ellipsoid scattered, rarely shortly linear (**Figure 3B, D, E, G**), while the nodules of *Sericanthe* are punctate to linear or branched along the mid-vein or scattered on leaves.

The genus *Psychotria* (syn. *Apomuria*) contains about 1850 trees, shrubs, subshrub, or liana species, distributing through tropical and subtropical regions. The *Psychotria* species with leaf nodule (about 80 species) are only found in Africa, mostly in southeastern part and surrounding islands.

The genus *Pavetta* comprises about 400 species of trees, shrubs, or subshrubs, distributing in Africa, tropical Asia, Australia, and Pacific islands. *Pavetta* contains about 350 species with leaf nodule, which is the largest number among the leaf nodulate genus. The leaf nodulate *Pavetta* species are found around the entire geographic range of the genus.

The African genus *Sericanthe* is composed mostly of shrubs, with about 21 species in southern and western Africa [57]. The genus *Sericanthe* was formerly referred to the genus *Neorosea*,

which was separated from the genus *Tricalysia* (see detail in [58]). Leaf nodules have been discovered in about 13 species of *Sericanthe*. The leaf nodules of *Sericanthe* are only visible on the abaxial side of the leaves.

The bacterial leaf nodules in Rubiaceae were first described as bacterial nodule and studied in 1902 [59]. In Rubiaceae, bacterial leaf nodule symbiosis was hypothesized to be obligate and cyclic in *Psychotria* and *Pavetta* [10]. In other words, the associated bacteria and the host plants cannot survive without each other, and the symbionts are retained in the host plant in all stages of its lifecycle. The symbiotic bacteria of the leaf nodulate rubiaceous plants are maintained in the mucilage secreted from dendroid colleters, a type of multicellular secretory trichome, in both apical and lateral buds [48, 60–62]. The nature and development of the leaf nodulate rubiaceous plants are briefly introduced below.

In the bud of *Psychotria* shoot apex, each pair of young leaves develops in a chamber formed by two pairs of stipules. The chamber is filled with mucilage that secreted by the branched colleters on the adaxial side of the stipules. The symbionts are maintained in the chamber and nurtured by the mucilage. During the leaf maturation, the bacteria enter the leaf tissue through precocious stomata on the abaxial side of young leaf and the sub-stomatal chamber begins to develop into a leaf nodule. As in *Ardisia*, the floral development in *Psychotria* is initiated from the mucilage-filled shoot bud [10]. The inflorescence primordium is enclosed by the chamber formed by circulate bracts with colleters adaxially. As each floret development, evidence shows that some mucilage is enclosed by the developing carpels and then the bacteria are eventually housed in the ovary. However, the detail mechanism of how the symbionts transferred to the embryos remains unclear in *Psychotria*. It was speculated that the bacteria may enter the embryo sac at the pollination stage where the bacteria are pushed into the embryo when the pollen tube penetrates micropyle.

In *Pavetta*, the symbionts are postulated to be maintained in shoot apex, leaf nodule, ovules, and seeds, but the complete life cycle of the bacterial symbiosis is not yet described [48]. The mechanism of bacteria maintenance in shoot buds and inflorescence primordium is similar to the case of *Psychotria*, also the nodule development in leaves. In *Pavetta*, the inflorescence buds are developed in the chamber formed by the circular stipules and immersed in the bacterial mucilage that secreted by colleters. As the floret develops, some bacterial mucilage is enclosed in the ovary and the bacteria are maintained by the mucilage secreted by the aril-like tissue at the base of each ovule. The details of how bacteria enter the embryo sac in *Pavetta* has not been observed and the same speculation as in *Psychotria* was made. The bacteria are also found in the mucilage around the cotyledons of the embryo in *Pavetta* seeds, which is failed to observe in *Psychotria*. However, the associated bacteria are found in the seedling of *Ps. kirkii*, suggesting the bacteria may retain in elsewhere of the seed rather than around the embryo [10].

Study of nodule structure and development of *Sericanthe* was only demonstrated in the species *S. andongensis* [63]. Mature nodules of *S. andongensis* are linear, and are localized on both sides of the petiole and mid-vein. The bud structure and nodule initiation of *S. andongensis* is similar to those of *Psychotria* and *Pavetta*. It is noteworthy that the “pseudonodules”, leaf nodules without bacteria inside, were observed in *S. andongensis*, as well as in *Ardisia* and

Pavetta species [25, 48, 63]. The results suggested the nodule in *Ardisia* and rubiaceous species could initiate the nodule development spontaneously rather than induced by the symbionts. Alternatively, the symbionts in this “pseudonodule” were present, but dead afterwards, or even may be digested by the host plants [27]. The complete life cycle of the leaf nodule symbiosis in *Sericanthe* is also unclear. It is not known whether the symbionts are present in the ovaries and/or the seeds. Thus, whether the symbiosis is cyclic in *Sericanthe* as that in *Pavetta* and *Psychotria* remains a question.

The structure and development of leaf nodule and the mechanisms of maintaining bacteria in shoot bud of the three genera in Rubiaceae are highly similar, which is a obvious result of convergent evolution. However, the opening the precocious stomata for bacterial infection of the leaves and the shape and distribution of the leaf nodules are different among the three genera. The stomata of leaves open adaxially in the process of nodule formation in *Pavetta* but open abaxially in *Psychotria* and *Sericanthe*. The shape and distribution pattern are variable between species, while a general pattern is described as above.

It is important to note that nodulation is not required in endophytic growth of bacteria in plants. Bacterial leaf endophytes are also found in non-nodulated Rubiaceae [64, 65], as well as in many angiosperms (such as in *Vitis* [66]). It is interesting that the endophytic *Burkholderia* was now known being widespread in the leaves of five non-nodulated rubiaceous genera, which are all in the tribe Vanguerieae of Rubiaceae [20, 21, 23]. None of these host plants showed an external sign of infection. The leaf endophytic *Burkholderia* was also found in non-nodulated *Psychotria* species [22]. The preference of the *Burkholderia* species forming a leaf endophyte association with rubiaceous plants is still a mystery, but definitely a key to understand the origin of the leaf nodule symbiosis.

4.2. The origin, phylogeny, and genomics

The ages of the origin of the leaf nodule evolution in *Psychotria*, *Pavetta*, and *Sericanthe* were estimated at about 9, 4, and 3 Mya, respectively [54, 67]. In *Psychotria*, the phylogenetic analyses showed ambiguous results by different authors that the leaf nodule evolved once or twice within the genus, and at least one secondary lost event was detected [22, 68]. Non-nodulated *Psychotria* forms an independent monophyletic clade in the genus, which is separated from the nodulated clade [22]. However, not all members in the clade harbors bacteria in their leaves, suggesting the non-nodulated *Psychotria*-*Burkholderia* association may be an unstable relationship between generations and/or individuals. In *Sericanthe*, the leaf nodule symbiosis may have a single origin, in spite of the phylogeny based on plastid genetic markers is poorly resolved [14]. A representative phylogeny of the members in *Pavetta* is not available so far, thus the origin and evolution in *Pavetta* is still unclear.

Horizontal gene transfer events occurred frequently between the leaf nodule symbionts of Rubiaceae. Both evidence of population genetics and whole genome study support the frequent genetic exchange hypothesis [19, 69]. However, the mechanism that how the symbiont exchanges their gene from the cyclic symbiosis system and how the association changes their partner is unknown.

The genome sizes of the sequenced symbiotic *Burkholderia* species from seven *Psychotria* species and a *Pavetta* species are around 2.4–6.2 Mb, which are relatively small in comparison of free-living plant-associated *Burkholderia* [19, 28]. All of these bacterial genomes contain large proportion of pseudogenes and transposable elements, referred to “eroded genomes”. Both the genomic size and composition indicate that the leaf nodule symbioses of rubiaceous plants are at an early stage of transition from free-living to host-restricted lifestyle. The genomic features are common in some recently evolved and vertically transmitted symbionts, such as the obligate cyanobiont of *Azolla filiculoides*, the bacterial symbiont *Serratia symbiotica* of pea aphids (*Acyrtosiphon pisum*) and conifer aphids (*Cinara tujafilina*) [9, 70, 71]. The essential housekeeping genes are mostly intact in the sequenced leaf nodule symbiont genomes despite of genome reduction, suggesting that these symbionts may not be dependent on the host for essential housekeeping functions.

The results of genomics, transcriptomics, and proteomics analyses revealed the capability of kirkamide synthesis in *Ca. B. kirkii*, the symbiont of *Ps. kirkii* [28, 72]. Kirkamide is a kind of C₇N aminocyclitol, which is a cytotoxin to insects and aquatic arthropods [73]. These results suggested that the leaf nodule symbionts may serve a defensive function to the host plants. Further studies sequenced genomes of the leaf nodule symbionts from seven *Psychotria* species and a *Pavetta* species, also showed the presence of these putative genes involved in kirkamide biosynthesis pathway [19]. However, many genes are not intact, that is, as pseudogenes, in the leaf nodule symbionts genomes, indicating that producing kirkamide might not be necessary for the host plants. These kirkamide synthetic genes are unique to the rubiaceous leaf nodule symbionts in comparison to the related *Burkholderia* species associated with other plants. Interestingly, the gene cluster is often located on a plasmid of the symbiotic bacteria, and sometimes flanked by transposon-like fragments, suggesting that these genes may be acquired from horizontal gene transfer [19]. With the current understanding from the genomic studies of the rubiaceous leaf nodule symbionts, the reasons for the seemingly obligate relationship still could not be readily answered.

5. Leaf gland symbiosis in Dioscoreaceae

The only case in monocots that bearing species with bacterial leaf gland symbiosis is found in Dioscoreaceae. This family is representing by the genus *Dioscorea*, the true yams, which comprises about 90% species of the family. Only one species, *Dioscorea sansibarensis*, was reported to have bacterial glands on leaf apices [10]. The Zanzibar yam, *D. sansibarensis* (syn. *Dioscorea macroura*), is a fast-growing vine that native to tropical Africa and Madagascar, and it is widely introduced and cultivated in many regions all over the world. *Dioscorea sansibarensis*, like most true yams, produces perennial underground tubers and aerial bulbils, which is the main reproductive organs of the species. Leaves of *D. sansibarensis* are large and roughly heart-shaped, with a conspicuously caudate apex or acumen (**Figure 3F, H**). Orr was the first who discovered the acumens are in fact full of bacteria and should be regarded as bacterial leaf glands [38].

The bacterial symbiosis of *D. sansibarensis* is not obligate because the plants can survive without the symbionts, and it is likely the host plants acquire the symbionts from environment, and the symbionts can also survive without the host plants for at least part of their leaf cycle [32]. Thus, it is not surprised that the associated bacteria in *Dioscorea* are so far the only leaf-nodule-associated species that can be cultured in ex situ condition. According to the microscopic view of the bacteria, the symbionts are non-motile, non-spore forming, ovoid-rod, Gram-negative bacteria [10, 16, 32]. Although several studies claimed they successfully cultured the symbiotic bacteria from *Dioscorea* in the past century [10], the true identity is not revealed until 2016 (i.e. *Oreella dioscoreae*, see Section 2.2). Surprisingly, the symbiont samples from various localities have been shown to be the same bacterial species [16]. It suggested that somehow the specificity still retains to a certain degree between the symbionts and *D. sansibarensis*.

The initiation and development of the bacterial symbiosis of the leaf glands has been studied in details in *D. sansibarensis* [32, 38–40]. The symbiosis first initiates during the development of the leaf acumen, which is apparently thicker than the leaf laminar in mature leaf. In the early stage of leaf growth and expansion, the margins of the acumen are swollen and bending inward to form an enclosed channel. The cavity becomes flask shaped as the two flanges develop. The central portion of the cavity then elevates to the level of the epidermis, separating the channel into two cavities. Secretory trichomes are also developing in the cavity and fill the whole space at this stage. The two cavities remain open to the external environment until the last stages of leaf maturation. In the maturation stage, the closed lumen is occupied by the mucilage secreted by the trichomes and the rapidly multiplied bacteria. Interestingly, the glandular acumen of *D. sansibarensis* is developed even the symbiotic bacteria are not present, but the glands are not as swollen as the bacteria-infected glands. Also, no mucilage is produced in uninfected glands and the trichomes in the gland lumen tend to degenerate. The results indicated that the development of glandular acumen is not induced by the symbiont, while the maintenance of the mucilage production and trichomes activity does require the cues from the bacteria. However, several questions remain obscure, for example, do the bacteria vertically transmitted in *D. sansibarensis*, if so, through seeds or bulbils? Also, do the plants acquire the bacteria from the environment in each generation, if so, how do the bacteria live with the facultative strategies in the dynamic environment?

In contrast to the leaf nodule symbiosis in other families, the bacteria-host relationship in Dioscoreaceae seems not so intimately associated. In Primulaceae and Rubiaceae, the bacteria are both harbored at the shoot apex and can be transmitted to the next generation through seeds; and, the symbiotic bacteria pass through the cuticle and invade plant tissue in some degree. However, neither the symbiont in Dioscoreaceae is found at the shoot apex, nor any invasion to host plant tissue is observed. Moreover, the symbiosis in Primulaceae and Rubiaceae are regard as obligate, while the association in Dioscoreaceae seems to be facultative. Therefore, the symbiosis of Dioscoreaceae was suggested to be a more primitive form of symbiosis than the leaf nodule symbioses in Primulaceae and Rubiaceae [32].

Dioscorea sansibarensis is by far the only known species with leaf gland in *Dioscorea*. There may be more *Dioscorea* species bearing the bacterial leaf gland symbiosis, such as *D. cochleari-apiculata*

and *D. dodecaneura* [10]. However, to our knowledge, no other bacterial leaf gland symbiotic species was formally reported except *D. sansibarensis*.

The genome of the symbiont of *D. sansibarensis*, *O. dioscoreae*, has been sequenced recently, which is about 5 Mb in size and is composed of a single chromosome without plasmid [16]. Based on the sequence data, the nitrogen-fixing-related genes are not found in the symbiont genome, so does the plant hormone gene associated with auxin or cytokinin biosynthesis or metabolisms. However, an ethylene signaling modulating gene, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, was identified in the genome. Some plant-associated bacteria can help plants to increase stress tolerance by producing the ACC deaminase, which can decrease the level of “stress ethylene” that inhibit plants growth [74, 75]. The discovery of the gene of ACC deaminase in the genome of *O. dioscoreae* provides clues for further study on the function of the symbiosis relationship to plants. Furthermore, genomic analysis showed similar features to many facultative anaerobic, free-living bacteria, and little effects of the interaction with host plants on the bacterial genome, suggesting the symbiosis association may be very young or facultative. It makes sense because the symbiotic bacteria of *D. sansibarensis* are epiphytes of phyllosphere in at least part of their life cycle.

6. Leaf gland symbiosis in Styracaceae

The leaf nodule or gland symbiosis has been known restricted in Primulaceae, Rubiaceae, and Dioscoreaceae for over a century. Until 2014, a newly found leaf-nodulated taxa was reported in *Styrax camporum* [11]. *Styrax*, known as storax or snowbell, is a small genus containing about 130 species of large shrub or small trees in the family Styracaceae. *Styrax* is mostly found to warm temperate to tropical regions in eastern and southeastern Asia and South America [76]. Stellate or peltate trichomes are common in *Styrax* species, while the glandular trichomes are rarely observed [76]. The glandular structure, sometimes refers to trichome, of *S. camporum* was found on young shoots and mature leaves, producing sticky secretion [11]. Unlike other cases of leaf nodule or gland which form swollen structures of part of leaf blade, the leaf gland of *S. camporum* is a glandular trichome-like structure with a nonsecretory short stalk and a secretory glandular body. The mature gland body is composed of a single layer of secretory cells around the axis. The actively secreting glands are distributed on top of leaf primordia and mature leaves. As the leaves maturation, the glands dry up and fall off except those on the petiole and leaf margin. Mature glands are turgid, irregular, pale yellow, and secreting mucilage, while the glands turn dark brown and shrink when senescence. The secreted mucilage covers the leaf primordia and young leaves at the shoot apex. Bacteria are observed immersed in the mucilage of the gland surface and intercellular space of the gland body. The associated bacteria are rod to ovoid shape with capsule. The sieve elements were observed in the stalk of the glands, which suggesting the transportation of some substances from the gland toward other tissues.

Unfortunately, little is known about the newly discovered leaf gland symbiosis in *Styrax*. The complete life cycle and nature of the bacterial symbiosis in *Styrax* remain obscure, as well as

the symbiont itself. For instance, does the bacterial leaf gland symbiosis also occur in other *Styrax* species? If so, does the associated bacteria specify to particular host species? Does the bacterial gland also occur on the reproductive tissue? If so, how do the glands develop on the reproductive tissue? Can the associated bacteria transmit to the seeds and seedling, as in *Ardisia* and *Psychotria*? If so, how? What is the main function of the symbiont serve to the host plants? In all, the identity and specificity of the associated bacteria and the function to the host plants are important issues, which are easy to achieve nowadays by the modern genome sequencing.

7. Conclusion, application, and future research

The leaf nodule symbiosis is the only case of the cyclic plant-microbe symbiosis with specialized structure in flowering plants. However, there are lots of knowledge gaps to be filled for such unique associations of plants and the symbiotic bacteria. The leaf nodule symbiosis is probably all cyclic in the examined species, except of *Dioscorea*, while only weak evidence was found in *Sericanthe* and *Styrax*. It is important to verify if it is true cyclic symbiosis in these cases, from the evolutionary aspects, and better our understanding on plant-microbe interactions. Three factors are necessary to confirm the presence of cyclic leaf nodule symbiosis. First, the symbiont should be able to maintain in the shoot apex through the nutrient-rich mucilage secreting from the specialized trichomes. Yet, having an enclosed chamber in the shoot apex is not necessary. Second, the symbionts could infect the young leaf and form mature leaf nodules or glands during the leaf development. Third, the symbionts have to enter the carpel and embryo, to form the symbiont-containing seeds. Despite much effort have been done by previous researchers, many details of the life cycle of these leaf nodule symbioses are still unclear, especially about the mechanisms of transferring the bacteria between different life stages of the host plant. The morphology of leaf nodules is usually diverse among species within the genus, except for the rather simple cases in *Dioscorea* and *Styrax*. The initiation and development of the leaf nodule symbiosis, however, are only observed in one or few species of each genus, leading to the questions on whether they are consistent between different species with different nodule morphologies.

Many symbiont genomes have been sequenced recently, but the interaction between the symbionts and host plants at molecular level is not well demonstrated. For instance, the mechanism of how the host plants prevent from the symbionts invasion and restricting the symbionts in the nodules or glands are unclear. The direct evidence of how the symbionts benefit to the host plants is also absent, and it is undoubtedly one of the most important knowledge for understanding the ecological evolution and agricultural application for the plants with the leaf nodule symbiosis.

In the leaf nodule symbiosis, the symbiotic bacteria retain in the plants cyclically as a permanently partner, which is a potential system for improving crops through genetic engineering and manipulating. In application, to design a plant-bacteria cyclic symbiosis system would be very useful for delivering the growth promoters, extra nutrients, pesticides, and so on, and the leaf nodule is even not necessary for the system. Several *Dioscorea* species are important

agricultural crops in tropical regions. The crop is threatened by various insect pests, fungi, viruses, and nematodes [77, 78]. The bacterial symbiosis in *Dioscorea* is a potential copartner for improving the crop against the pathogens. In *D. sansibarensis*, the bacteria are culturable, which means to modify bacterial genome or to insert particular gene fragments are feasible. For instance, inserting the pesticide synthetic genes to against the pathogens and herbivores may elevate the yield and lower the cost of pesticide using by farmers. In addition, it could also lower the environmental concerns often raised by the GMO crops since the crop itself does not contain a modified genome. In *Dioscorea*, the bacterial symbiosis is so far only found in *D. sansibarensis*, but it is possible that the same association will be found in other *Dioscorea* crop species or the bacteria could be used to infect other crop species.

In *A. crenata*, one of the peptide with biomedical application was found in fact contributed by the leaf nodule symbionts [29]. To the authors experience, *A. crenata* grows slowly and the growth condition is somewhat demanding. If the symbiont could be isolated and cultured in the future, synthesizing and purifying the peptide will be much faster and space-efficient. Even if the symbiont could not be cultured after all, the gene cluster can be inserted to another operable and culturable bacteria. The results might be able to benefit other *Ardisia* species since many of them are important Chinese medicinal plants [79].

The genomics of the leaf nodule symbionts have been studied in most genera, except of *Sericanthe* and *Styrax*. However, many questions are awaiting to be answered with the symbiont genomic sequences. One of the most interesting questions is the hypothetical “obligate symbiosis” relationships in *Ardisia* and rubiaceous plants. The symbionts genomes give no explanation for the dependence of the symbionts from the host plants. Clearly, there are more to be explored with the genomic or metabolomics data.

The cause of the leaf nodule initiation and development, as well as the maintenance of the symbionts in the shoot bud is poorly known at molecular level so far. In *Dioscorea*, the leaf gland seems being able to develop spontaneous without the symbionts cue. However, the cues of the leaf nodule development in other nodulate plants are poorly known. In rubiaceous nodulate plants, the “pseudonodule” has been observed, but the formation of “pseudonodule” may be caused by fading out of the symbionts. To compare the genomics or transcriptomics data of nodulated *Psychotria* and non-nodulated *Psychotria* is a possible method to find out the candidate genes that regulating the leaf nodule development and the mucilage-secreting trichomes, which are important determinants of the cyclic leaf nodule symbiosis. Taken these together, we shall be able to shed light on the intriguing phenomenon of symbiosis between the plants and the bacteria lived intimately in their leaves.

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