Genetic analysis of ethylene regulation of legume nodulation

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The gaseous hormone ethylene has multiple roles in plant development and responses to external cues. Among these is the regulation of 'Rhizobium'-induced nodulation in legumes. Extensive descriptive literature exists, but has been expanded to allow more mechanistic analysis through the application of genetics. Both mutants and transgenics displaying ethylene insensitivity have now been described, suggesting an intimate interplay of ethylene response, plant development and nodulation.

Nodulation of legumes is significant as the nitrogen fixing symbiosis adds critical available nitrogen to the biosphere. Often major crop plants are either legumes (c.f., soybean, peanut, bean, medics, clovers, chickpea), or they rely on rotational practices involving legumes. Nodule formation occurs predominantly on roots after mitogenic stimulation of cortical and pericycle cells by the Rhizobium-secreted lipo-oligosaccharide Nod-Factor. To date, the timing, the signal, its receptor (a LysM receptor kinase complex), major parts of the down-stream response cascade (involving ion channels, a calcium calmodulin dependent protein kinase, a cytokinin receptor and numerous transcription factors) and the target cell types are known[1,2] (**Fig. 1A**). As a result, nodule ontogeny is an elegant and mature experimental system to analyse plant development, associated signaling and behavior.[3] Genetic, genomic, physiological and biochemical tools are currently being combined to optimise the role of legumes in diverse agricultural and silvicultural systems to facilitate more sustainable production of food, feed and biofuels.

Mutant and Transgenic Analysis using Model Legumes

Ethylene gas exposure, or growth in the presence of ethylene precursors such as ACC, limits nodule numbers per plant through reduced infection by 'rhizobia'[4-6] and decreased ability to initiate cell divisions in target plant zones behind the growing root tip (the 'zone of nodulation' characterized by emerging root hairs). The laboratory of Doug Cook and Varma Penmetsa was first to describe legume mutants with increased insensitivity for the classical 'triple response' during dark germination.[7] Specifically, a *Medicago truncatula* mutant, called 'sickle', because of the characteristic root deformation caused by excessive nodule initiation, was described. Of symbiotic significance, this recessive mutant displayed increased nodule initiation and rhizobial infection. Its symbiotic phenotype was controlled by the root as shown by reciprocal grafts. Recently, the affected gene was cloned and shown to be *EIN2*,[8] a known member of the signal response chain to ethylene in *Arabidopsis thaliana*. *EIN2* is presumed to encode a nuclear membrane component, possibly transmitting a cytoplasmic response in CTR1 to nuclear transcription and response factors.

Recently, Lohar et al.[9] reported a similar increased nodulation phenotype in the model legume *Lotus japonicus*. In contrast to Cook's work, Lotus nodulates in essentially the same steps but the induced nodule primordium has a short life span leading to determinate nodule formation (**Fig. 1B**); in contrast, Medicago, like pea and clovers, forms indeterminate nodules characterized by a more persistent (but still terminal) meristem (**Fig. 1C**). Hence, one finds the distinctive difference of spherical (determinate) and cylindrical nodule shapes (indeterminate).

The Lotus ethylene insensitive lines were constructed by transgenic modification using the dominant Arabidopsis *ETR1-1* mutant gene.[10] *ETR1* encodes a two-component histidine kinase receptor, which if mutated prevents ethylene action.[11] It most likely functions in the ER membrane. *AtETR1-1* was introduced as a single copy under the control of the constitutive *35S CaMV* promoter.[12] Results were confirmed over several generations with independent lines. Strong ethylene insensitivity was detected in germination tests conducted in the dark. Classical ethylene insensitivity phenotypes included extensive hypocotyl length in triple response assays, reduced number of lateral roots, flower petal retention and delayed flowering and fruit ripening (**Fig. 2A and E**). Additionally, increased nodule numbers (both in the presence and absence of ACC) (**Fig. 2H**), disrupted root hair infection and epidermal cell divisions (**Fig. 2C and D**), altered radial distribution of nodule primordium induction (normally almost exclusively off the xylem pole, but now with an eight-fold increase off
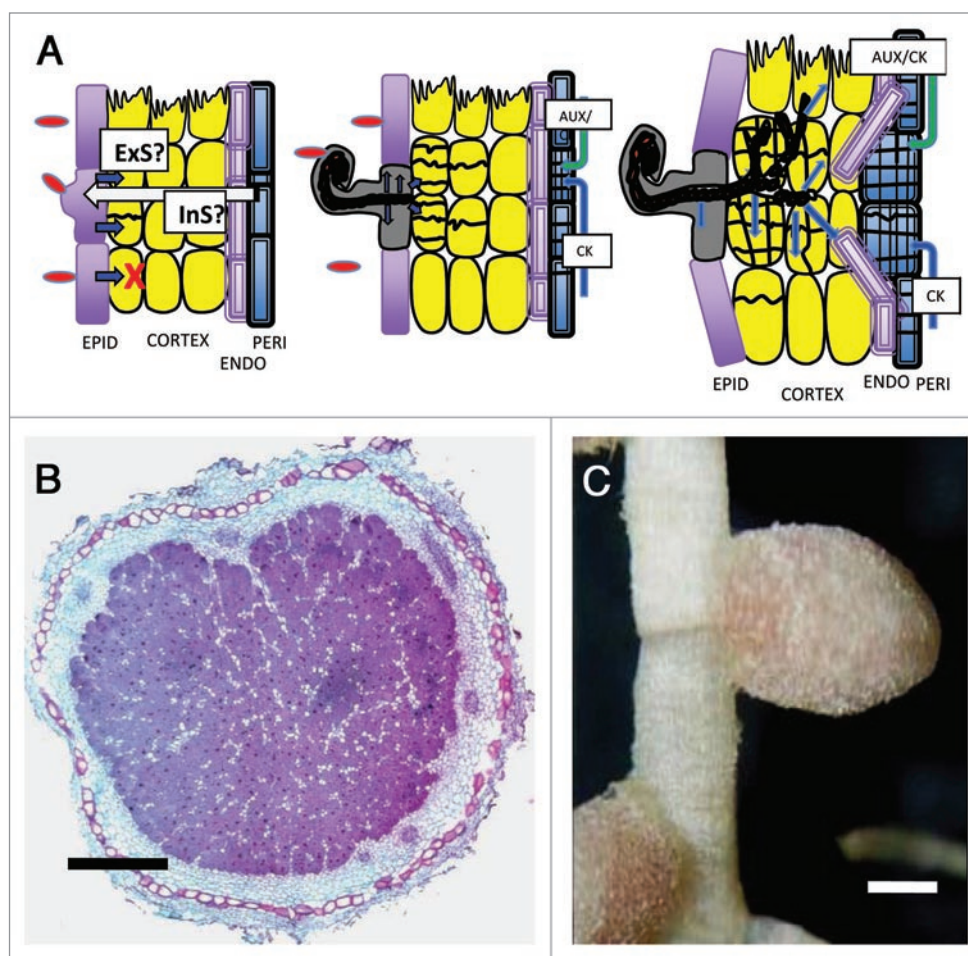
Figure 1. Critical stages of nodule initiation in determinate nodulating legumes. (A) Rhizobium cells in the soil recognize plant-secreted flavones and attach to the epidermal (EPID) cells. Nod-factor, a lipo-oligosaccharide decorated with various host-range determining moieties, is perceived by the legume epidermal cells which respond in two ways, namely re-arrangement of the cytoskeleton resulting in root hair curling and bacterial entrapment, and induction of ectopic cytokinin signalling (possibly synthesis). These events allow (1) bacterial invasion through an infection thread (as inverted root hair cell wall) and (2) generation of an unknown external signal (ExS) that activates cell division in subepidermal cortical cells [in harmony with an unknown internal signal (InS)]. Blue arrows signify continued Nod Factor stimulation. Note that in indeterminate nodulators such as clover and Medicago, these cell divisions occur in the deep cortex adjacent to the endodermis. These cell divisions occur predominantly (90–96%) opposite xylem pole cells, which is altered in ethylene insensitive mutants/transgenics. The vascular system of the root extends into the nodule to provide hormonal and nutritional transport. An early nodule primordium is deemed non-autonomous, while the more advanced cell cluster is able to generate most of its own hormones for cell proliferation. EPID, epidermis; PERI, pericycle; ENDO, endodermis. The final nodule is a chimeric organ having arisen from both cortical and pericycle origins. (B) cross-section of a fully nitrogen-fixing nodule of soybean (photo: Lisette Pregelj, CILR), and (C) pea (*Pisum sativum*; photo: Dr. Alex Borisov, St. Petersburg). Bar = 1 mm.

the phloem pole; **Fig. 2F and G**), twisted hypocotyl (**Fig. 2B**), and increased number of bacteroids per symbiosome (two- to three-fold) were observed.

The Lotus *ETR1* transgenics nodulated in the presence of inhibitory levels of ACC, but interestingly still were inhibited in primary root growth. This may suggest that the 35S CaMV promoter is not active in the appropriate cell types, or that another receptor (e.g., ERS1) may be critical for root meristem activity. It also suggests that root growth responses and nodulation, although both involving the same target tissues (i.e., the pericycle close to xylem poles) respond in opposing ways (see **Fig. 1**).

Of interest is a previously unrecognized root hair invasion and cell division response. At a high frequency, root hairs in *LjETR1-1* seedlings were filled with bacterial infection threads that did not penetrate into the cortex (**Fig. 2C and D**). At times these epidermal

cells divide repeatedly, but without cell enlargement. One speculates that increased Nod Factor production, concomitant ectopic cytokinin synthesis, and altered plant 'immune response', leading to a pseudo-hypersensitive response, cause this abnormal cellular response.

Lotus transgenics, like *sickle* in Medicago, had wild-type sensitivity to nitrate inhibition of nodulation, suggesting that although nitrate treatment and ethylene responses in legumes are well-documented negative regulators, they do not act in the same pathway.

Similar results were obtained by Nukui and associates in Japan, who transformed Lotus with the less-characterized ethylene *ERS1* receptor gene from melon.[13] Although these results suggested a broader commonality in ethylene regulation of nodulation, some recent results suggest further complexity. Again EMS-induced ethylene insensitive mutants of *L. japonicus* were isolated using a Triple

Response seedling assay.[14] Mutant *ENIGMA-1* was isolated together with a second independent, but allelic mutant *(ENIGMA-2)*. Both exhibited recessive, root-controlled phenotypes. Both mutants had reduced nodulation (about 70–75% of wild type Miyakojima), yet both are mutated in *LjEIN2*! Yet ethylene receptor transgenics of Lotus (i.e., *LjETR1-1*) showed increased nodulation. All mutants and transgenics possessed strong ethylene insensitivity phenotypes in other developmental processes, supporting the physiological functionality of the genetic alteration. Thus, a paradox exists as the *EIN2* mutant of Medicago has increased nodule number whilst an *EIN2* mutation in Lotus does not.

Ethylene and Autoregulation of Nodulation

Legumes also control nodule numbers through a systemic process labeled "Autoregulation of Nodulation (AON)".[15] Mutants in this circuit have a supernodulating or hypernodulating phenotype.[16] Such mutants were isolated in several legumes, though most advances have so far been made with soybean and Lotus. All AON-deficient mutants display decreased nitrate sensitivity to nodulation. One causative gene is the CLAVATA1-related LRR receptor kinase NARK (nodule autoregulation receptor kinase[17]). The equivalent gene in Lotus is called *HAR1* (hypernodulation and aberrant root[18,19]), and in *Medicago truncatula* is called *SUNN* (supernummary nodulation[20]). Other loci such as *KLAVIER*, *ASTRAY* and *TOO MUCH LOVE* exist in Lotus and have been partially characterized. Significantly, *GmNARK* was shown to control nodulation through its expression in the shoot vasculature[21,22] and this was confirmed in other legumes.[18,19,23] NARK interacts with a protein phosphatase (KAPP[24]), reminiscent of the interaction seen by CLAVATA1 in the control of the apical meristem.

One presumes that multicellular plants utilise a complex set of LRR receptor kinases to control illegitimate cell divisions; i.e., NARK (and its cousins SUNN and HAR1) are de facto 'tumor suppressors'.[25] Short and long distance signaling molecules are presumed[26] and have been isolated as CLE-like peptide-encoding genes (no binding studies yet) in both *L. japonicus*,[27] Medicago (Holsters M, Ghent, personal communication) and soybean (Reid D and Ferguson B,
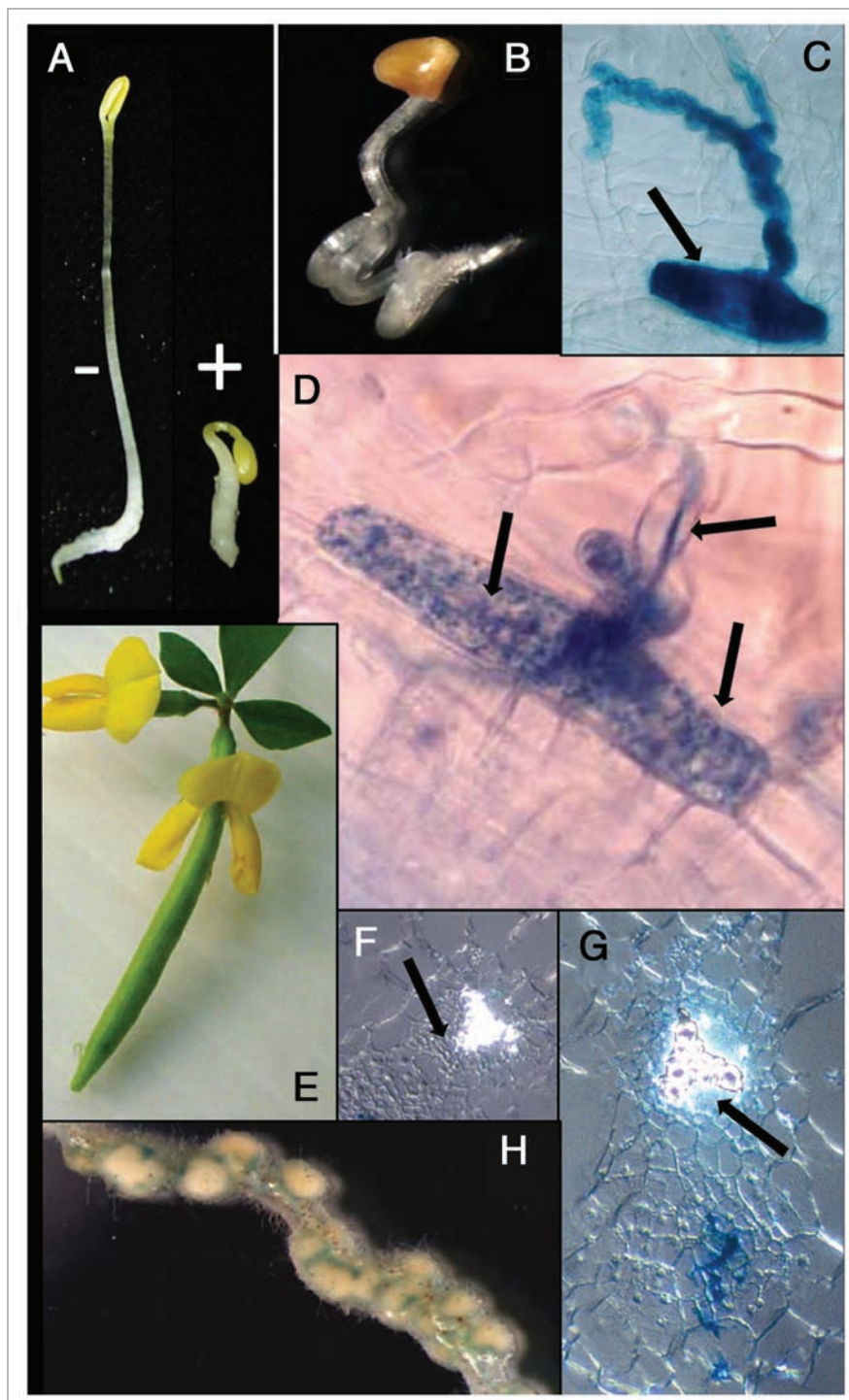

Figure 2. Ethylene-associated phenotypes. (A) classical triple response of dark grown *Lotus japonicus* wild-type seedlings grown with (+) and without (-) added ethylene or its precursor ACC; (B) ethylene super-insensitive seedling of line *LjETR1-1* with a twisted hypocotyl; (C) Infected root hair but the infection was blocked in transgenic line *LjETR1-1* (X-gal staining; *Mesorhizobium loti* strain NSP2235::*lacZ*). (D) same as (C), but illustrating epidermal cell divisions (note nuclei). (E) Flower with retained petals and developing seed pod. (F) Root section of wild type showing nodule initiation (lower left) off the xylem pole (see arrow); (G) as (F) but *LjETR1-1*, but cell division cluster off the phloem pole (see arrow); (H) nodulated Lotus *LjETR1-1* root portion showing high density nodulation.
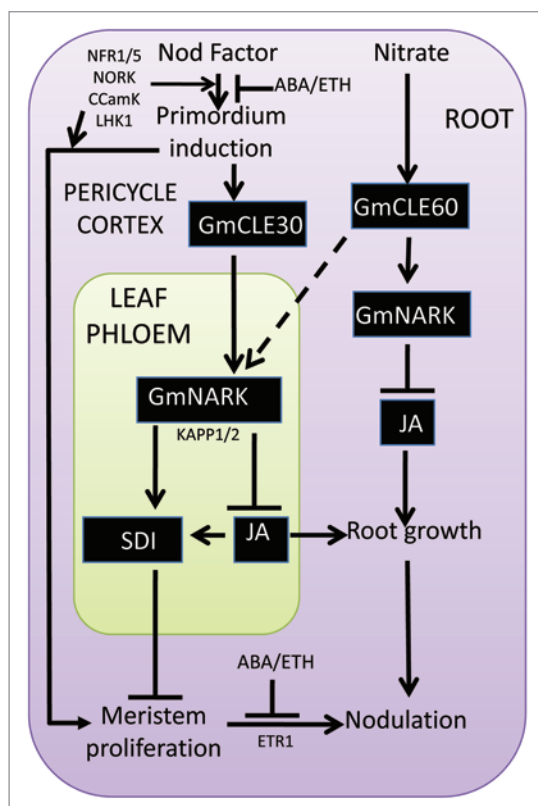
Figure 3. Model for nodule initiation, local nitrate inhibition and systemic AON control circuits. We propose that Nod factor induces cell proliferation that also leads to production of a CLE-type peptide which either in a complexed form or as a prepeptide travels to the leaf where it interacts with the CLAVATA1-like LRR receptor kinase NARK (or HAR1/SUNN). It is unclear whether jasmonic acid synthesis and response transcript changes are directly or indirectly controlling the release and transport of the SDI (shoot-derived inhibitor), thought to be a small molecular weight substance (possibly a metabolite or its analogue), which activates nodule meristem arrest in the root. The same compound is proposed to be a positive regulator of root meristem. ABA and ethylene inhibit nodulation possibly prior to calcium spiking during Nod Factor perception.[6] A secondary role (at least for ethylene) is proposed via radial potentiation for cell divisions of cortical cells. In contrast nitrate activates a related peptide that also is proposed to interact with NARK but only in the root, leading to localised inhibition of nodulation.

unpublished data; see **Fig. 3**). It is presumed that the LRR domain is the major binding site for a CLE peptide. Genome-wide expression analysis suggests that root inoculation with Bradyrhizobium via NARK negatively controls the biosynthesis of jasmonic acid in the leaf vasculature.[28] A common phenotype in all AON-defective mutants is a partial insensitivity to otherwise inhibitory levels of nitrate, and reduced root systems (with varying degrees of penetrance as Lotus *har1* mutants tend to be severely affected, while soybean and Medicago show the response only when extensively supernodulated).

Interactions and Cross-Talk

A key question is how does AON interact with ethylene and cytokinin signaling? Does cell division control involve the cytokinin receptor (CRE1/LHK1) known to engage in cross-talk with the related ethylene receptor cascade? Several results permit the conclusion that the AON and ethylene pathways are independent. First, ethylene mutants do autoregulate as shown by their suppressed nodulation pattern below the heavily nodulated region (the 'sickle'). Second, both soybean and Lotus AON mutants are still ethylene sensitive.[29] Third, double mutants of *Mtsunn/Mtsickle* and *Ljhar1/LjETR1-1* do not show an additive phenotype.[29,30]

However, the jury is still out. *Ljhar1-1* mutants possess a strong root phenotype.[31,32] Seedling primary root growth and lateral initiation are affected. When mutant and wild-type seedlings are treated with inhibitory levels of the cytokinin BAP, root growth is severely inhibited. Similar findings have been reported using *Pisum sativum*.[33,34] In Lotus, this inhibition is reversed by treatment with AVG or silver ions (both ethylene action inhibitors).[31] However, recovery to near wild-type levels of root length occurred preferentially in the *har1-1* mutant. We tested this phenomenon, not with phytohormone inhibitors, but by mutant interactions. Confirmed double mutants of *Ljhar1-1* and *LjETR1* (HE11 and HE19,[35]) were tested for their root and nodulation phenotypes. Of great interest was the fact that the presence of the ethylene insensitivity receptor suppressed the primary root growth inhibition caused by the absence of the *LjHAR1* gene product. Skilled reciprocal grafting of both HE11 and HE19 (*har1; ETR1-1*) to the *Ljhar1-1* mutant created plants that harbored a homozygous *har1-1/har1-1* condition in the entire plant, but with the *ETR1-1* controlled ethylene insensitivity being active in either shoot or root.[30] We conclude that the suppression of the *Har1* deficiency induced root dwarfing required ethylene insensitivity in the shoot. We propose more complex signal exchanges exist between the root and the shoot in both the control of nodulation and root growth[36] (**Fig. 4**).

Conclusions

Using transgenics and induced mutants it was possible to dissect mechanisms of ethylene action as it relates to nodule establishment and systemic nodulation control. Paradoxes were revealed, possibly hinting at yet unknown complexities arising from our present ignorance pertaining to hormonal action differentiating determinate (spherical) and indeterminate (cylindrical) nodules (**Fig. 1B and C**). Clearly regulation of nodule meristem activity is 'in play'. Further analysis utilizing new functional genomic tools, coupled with attention to cell-specificity and metabolites (as compared to transcript profiling) will be needed. One can be certain that the ethylene story in legume nodulation will reveal many new facets of plant biology, especially concerning cross-talks and interactions.
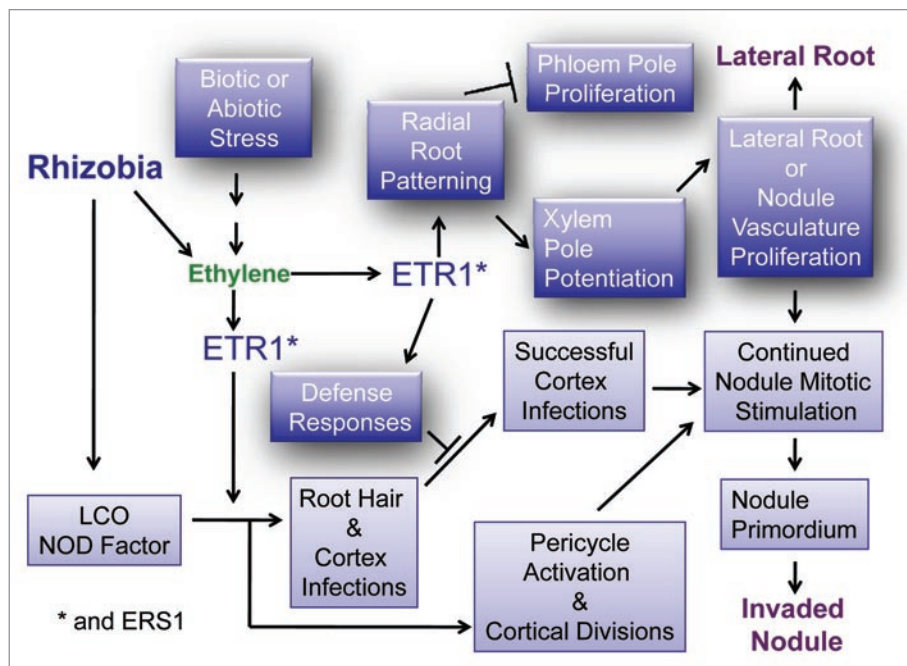
Figure 4. Conceptual diagram of inter-relations of ethylene regulation during nodulation and root growth. Ethylene works through balanced signalling on root and nodule meristems. Additionally the 'plant immune response' is activated leading to concomitant biochemical changes such as alterations of the phenylpropanoid pathway. These in turn affect 'rhizobia' as well as endogenous plant processes such as hormone translocation. Mutations in the *ETR1* receptor gene are complex to analyse as it is unclear what tissue specificity and distribution this receptor has relative to related receptors like ERS1.

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