Functional analysis of ATP-binding cassette proteins involved in nodule formation in legume plants

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Legume plants have an ability to fix atmospheric nitrogen into nutrients via symbiosis with soil microbes. As the initial event of the symbiosis, legume plants secrete flavonoids into the rhizosphere to attract rhizobia. The secretion of flavonoids is indispensable for the establishment of symbiotic nitrogen fixation, but almost nothing is known about the membrane transport mechanism of flavonoid secretion from legume root cells. I first performed biochemical analyses to characterize the transport mechanism of flavonoid secretion using soybean (*Glycine max*) and genistein, which is present in the root exudates of soybean to induce *nod* genes of *Bradyrhizobium japonicum*, as a model system.

Plasma membrane vesicles were purified by fractionation of microsomes prepared from soybean roots on a discontinuous sucrose density gradient. Antibodies against plasma membrane H^+-ATPase, vacuolar pyrophosphatase and luminal binding protein were used as markers of plasma membrane, tonoplast, and ER membrane, respectively, in western blot analysis. Plasma membrane vesicles were recovered from the interface of 30 and 40 % sucrose layers. The time course of genistein transport by plasma membrane vesicles was measured. The genistein transport, which was critically dependent on the presence of MgATP, linearly increased up to 15 min incubation. The pH dependency of genistein transport with Tris-Mes buffer ranging from pH 6.0 to pH 9.5 shown its optimum at around pH 8.0, but appreciable activity was observed within the tested pH range. Mechanism of ATP-dependent genistein transport at the plasma membrane vesicles was investigated using various transport inhibitors. Vanadate, a typical inhibitor of ABC transporter acting as a phosphate analogue, inhibited ca. 60 % of the genistein transport, while various ionophores such as gramicidin D, nigericin, or valinomycin did not inhibit the genistein transport (Figure 1). These results suggest the possible involvement of an ABC-type transporter in the secretion of flavonoids from soybean roots [1]. Competition experiments using various flavonoids of both aglycone and glucoside varieties suggested that this transporter recognizes genistein and daidzein, another signaling compound in soybean root exudates, as well as other isoflavonoid aglycones as its substrates. In order to assess whether or not the genistein secretion of soybean roots is induced under nitrogen starvation conditions, transport activity of membrane vesicles from soybean roots grown with or without supplementary nitrogen was compared. Membrane vesicles prepared from plants under nitrogen starvation exhibited a slight, albeit not clearly statistically significant, increase in the genistein transport activity. These findings suggest that transport activity was constitutive regardless of the availability of nitrogen nutrition.

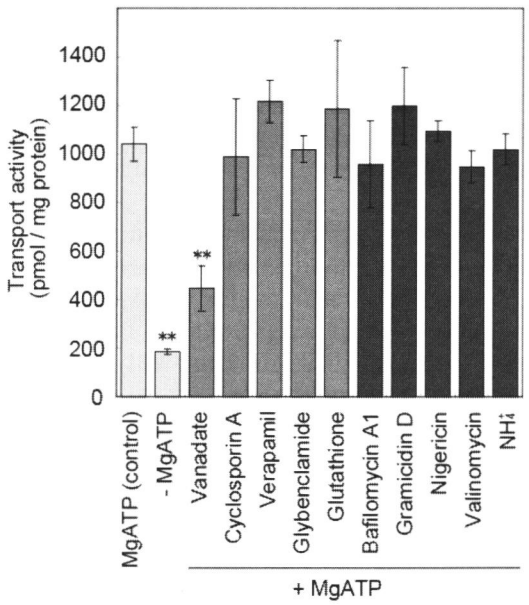

Figure 1. Effects of inhibitors on the genistein transport

Genome-wide analysis of ABC protein genes in a model legume plant, *Lotus japonicus*, was then carried out. For analysis of the *Lotus* genome sequence, a new method "domain-based clustering analysis" was devised, where domain structures like the nucleotide-binding domain and transmembrane domain, instead of full-length amino acid sequences, are used to compare phylogenetically each other. This method enabled to characterize fragments of ABC proteins which frequently appear in a draft sequence of the *Lotus* genome. Ninety-one putative ABC proteins in *L. japonicus*, i.e. 43 "full-size", 40 "half-size" and 18 "soluble" putative ABC proteins were identified (Table 1) [2]. The characteristic feature of the composition is that *Lotus* has extraordinarily many paralogues similar to AtMRP14 and AtPDR12, which are at least six and five members, respectively. Expression analysis of these genes revealed the putative involvement of AtPDR12-like genes in the nodulation process.

Table 1. Comparison of the ABC protein superfamily in plants and humans

	Genome	A	B	C	D	E	F	G	SMC	I	Total	Reference
Lotus	450 Mb	3 (2)	15 (3)	17	2 (1)	1	6	36 (24)	1	10	91	this work
Arabidopsis	125 Mb	17 (16)	27 (5)	15	2 (1)	2	5	44 (29)	4	15	131	3, 4
Rice	440 Mb	7 (7)	28 (4)	17	3 (1)	2	5	51 (30)	4	10	125	5
Human	3000 Mb	12	11 (7)	13	4 (4)	1	3	5 (5)	0	0	49	6

Parentheses indicate the number of half-size ABC proteins for the subfamilies, in which both full- and half-size members are classified as one group.

An AtPDR12-like gene, named LjPDR1, was chosen for further analyses. LjPDR1 is expressed specifically in roots and nodules, and induced by methyl jasmonate. Shoot-applied methyl jasmonate strongly (ca. 800-fold) induced the expression of LjPDR1 in roots. Histochemical analyses with a chimeric gene of LjPDR1 promoter and β-glucuronidase showed the strong expression of LjPDR1 in lateral roots (around the vascular bundles), lateral root primordia, nodule primordia, young nodules, and root tips. Membrane fractioning with sucrose density gradient and aqueous two-phase partitioning system revealed the localization of LjPDR1 at the plasma membrane. These results suggest that LjPDR1function in the nodule formation process mediating the loading of metabolites into vessels or sieve tubes.

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