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Development and Partial Characterization of Nearly Isogenic Pea Lines (*Pisum sativum* L.) that Alter Uptake Hydrogenase Activity in Symbiotic *Rhizobium*[1]

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ABSTRACT

Some *Rhizobium* bacteria have H_2-uptake (Hup) systems that oxidize H_2 evolved from nitrogenase in leguminous root nodules. Pea (*Pisum sativum* L.) cultivars 'JI1205' and 'Alaska' produce high Hup (Hup^{++}) and moderate Hup (Hup^{+}) phenotypes, respectively, in *Rhizobium leguminosarum* 128C53. The physiological significance and biochemical basis of this host plant genetic effect are unknown. The purpose of this investigation was to advance basic Hup studies by developing nearly isogenic lines of peas that alter Hup phenotypes in *R. leguminosarum* strains containing *hup* genes. Eight pairs of nearly isogenic pea lines that produce Hup^{++} and Hup^{+} phenotypes in *R. leguminosarum* 128C53 were identified in 173 F_2-derived F_6 families produced from crosses between JI1205 and Alaska. Tests with the pea isolines and three strains of *hup*-containing *R. leguminosarum* showed that the isolines altered Hup activity significantly ($P \leq 0.05$) in 19 of 24 symbiotic combinations. Analyses of Hup phenotypes in F_6 families, the F_1 population, and two backcrosses suggested involvement of a single genetic locus. Three of the eight pairs of isolines were identified as being suitable for physiological studies, because the two lines in each pair showed similar growth, N assimilation, and flowering traits under nonsymbiotic conditions. Tests of those lines under N_2-dependent conditions with isogenic Hup^{+} and negligible Hup (Hup^{-}) mutants of *R. leguminosarum* 128C53 showed that, in symbioses with Hup^{+} rhizobia, two out of three Hup^{++} pea lines decreased N_2 fixation relative to Hup^{+} peas. In one of those cases, however, the Hup^{++} plant line also decreased fixation by Hup^{-} rhizobia. When results were averaged across all rhizobia tested, Hup^{+} pea isolines had 8.2% higher dry weight ($P \leq 0.05$) and fixed 12.6% more N_2 ($P \leq 0.05$) than Hup^{++} isolines. Pea lines described here may help identify host plant factors that influence rhizobial Hup activity and should assist in clarifying how Hup systems influence other physiological processes.

Rhizobium and *Bradyrhizobium* bacteria in leguminous root nodules can contain two enzyme systems involved in H_2 metabolism. One, the nitrogenase complex, simultaneously reduces N_2 to NH_3 and protons to H_2 (8). The other, a Hup[4] system that oxidizes H_2 to water, was first reported in *Rhizobium leguminosarum* bacteroids from pea root nodules (27). Today it is known that many, but not all, rhizobia express a Hup^{+} phenotype (26). Possible advantages of H_2 oxidation in symbiotic rhizobia include protection of nitrogenase from O_2 and H_2, as well as the production of ATP and reductant.

Rhizobial Hup systems have been characterized most thoroughly in *Bradyrhizobium japonicum*. Information is available from analyses of *hup* genes in symbiotic bacteroids (16, 28), from physiological genetic studies of Hup activity in free-living cells (22), and from direct purification of the uptake hydrogenase (2) and a possible electron transport component of the Hup system (13) in bacteroids. Despite these advances, no complete description of proteins required for transfer of electrons from H_2 to O_2 in either free-living or symbiotic *B. japonicum* cells is yet available (26). Consequently, it is not understood how changes in electron fluxes of the Hup system may affect other, possibly related processes, such as N_2 reduction and oxidative phosphorylation. Less is known about the Hup^{+} phenotype in *R. leguminosarum*, but genes responsible for Hup in that organism occur on the pSym plasmid (7) and often show homology to *hup* genes from *B. japonicum* (20, 24).

In symbiotic rhizobia, the Hup system is affected by the host plant. Thus, environmental factors perceived by the plant, such as irradiance (6) and salinity (18), alter Hup activity. Unexplained effects of host plant genotype on Hup activity also occur in different plant species nodulated by the same rhizobia (10, 12, 19, 21, 23). Obviously, the physiological effects of Hup activity in a single strain of *Rhizobium* are difficult to interpret when the host plants are members of different species. Plants more suitable for physiological comparisons have been identified in *Pisum sativum* L. cultivars which produce Hup^{++}, Hup^{+}, and Hup^{-} activity in a single strain of *R. leguminosarum* (3, 5). Such effects on Hup activity in root nodules generally can be detected indirectly by measuring the RE of N_2 fixation, which is calculated as RE = 1 − (H_2 evolved in air/C_2H_2 reduced) (29). Any putative differences in Hup activity measured by this technique, however, must be confirmed with direct assays of H_2 uptake.

Clarifying the biochemical functioning and physiological significance of the Hup system in leguminous root nodules

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[4] Abbreviations: Hup, H_2 uptake; Hup^{++}, high Hup; Hup^{+}, moderate Hup; Hup^{-}, negligible Hup; RE, relative efficiency of N_2 fixation.

requires data on genetic factors in both symbiotic partners. The present study was initiated to characterize plant genetic factors that alter rhizobial Hup activity and to develop nearly isogenic pea lines that produce Hup^{++} and Hup^{+} phenotypes in the *hup*-containing *R. leguminosarum* strain 128C53 (*i.e.* Hup^{++}/Hup^{+} pea isolines).

MATERIALS AND METHODS

Organisms

Pea (*Pisum sativum* L.) materials identified as cultivars 'JI1205' (Hup^{++}) or 'Alaska' (Hup^{+}) were grown from single seed selections in lines with the same names which were supplied originally by B. Snoad (John Innes Institute, Norwich, Norfolk, UK) and Burpee Seed (Riverside, CA), respectively. Hup phenotypes in the two cultivars were determined with *Rhizobium leguminosarum* 128C53 (3, 5) because a genetic basis for Hup activity in *R. leguminosarum* was first established with this strain (7) and isogenic Hup^{+}/Hup^{-} mutants are available (9). All genetic crosses were made with emasculation, and the success of hybridization was assessed by following the recessive tendrilless trait from JI1205. Data for H_2 evolution, $^3H_2$ uptake, and C_2H_2 reduction were obtained from plants grown in microbiologically controlled, 750-mL Leonard jars with vermiculite under growth chamber conditions supplying a 16/8-h light/dark cycle, 21°C/15°C, 50% RH, and a photosynthetic photon flux density (400–700 nm) of 500 $\mu E \cdot m^{-2} \cdot s^{-1}$ (3). Tests of growth and N assimilation were run with 750-mL Leonard jars in a glasshouse with maximum and minimum temperatures controlled at 30 and 15°C, respectively. Plants received one of two nutrient solutions: a) a standard N-free solution for N_2-dependent growth (3) or b) the N-free solution supplemented to contain 8 mM NH_4NO_3. All N_2-dependent plants were inoculated with *R. leguminosarum*.

R. leguminosarum strains used in this study are described in Table I. Strains 128C30 and 175R1, which had different levels of Hup activity on 'Homesteader' pea (25) and patterns of hybridization with *hup*-specific DNA that differed from 128C53 (24), were generously supplied by Dr. L. M. Nelson, NRC, Saskatoon.

Physiological Assays

H_2 evolution in air (10 min) and C_2H_2-dependent C_2H_4 evolution (5 min), referred to as C_2H_2 reduction, were measured sequentially on excised root systems (3) to calculate RE (29). Hup activity of root nodules was measured directly as $^3H_2$ incorporation into H_2O after blocking H_2 evolution from nitrogenase for 5 min with C_2H_2 (6, 9). JI1205 and Alaska root nodules, blanks, ^3H_2O standards, and unnodulated roots were run as controls in each $^3H_2$-uptake test.

Table I. *Characteristics of Rhizobium leguminosarum Strains Studied*

Strain	Characteristics	Hup Phenotype[a]	Reference
128C53	Field isolate	Hup^{+}	7
128C30	Field isolate	Hup^{+}	25
175R1	Field isolate	Hup^{+}	25
3855	128C53 Str^r	Hup^{+}	9
518	3855pRL6JI::Tn*5-mob*	Hup^{+}	9
523	3855pRL6JI::Tn*5-mob*	Hup^{-}	9
524	3855pRL6JI::Tn*5-mob*	Hup^{-}	9

[a] Hup phenotype assessed on Alaska pea for this study.

Plant and Data Analyses

Plant dry weights were measured after 48 h at 70°C. Reduced N content was determined by fluorometric detection of ammonium in digested samples (15). Data were analyzed by standard statistical methods (32).

RESULTS

Development of F_6 Families

A random F_3 seed was taken from each of 193 F_2 plants produced from crosses of JI1205 (Hup^{++}) and Alaska (Hup^{+}) (97 JI1205 × Alaska; 96 Alaska × JI1205) under glasshouse conditions. The F_4 and F_5 generations also were developed from a random seed in each of the separate lines (single seed descent method). Sufficient seeds (≥ eight) for analyses of RE and Hup phenotypes in F_6 families were obtained from only 173 F_2-derived F_5 lines.

Analyses of F_6 Families

Preliminary tests with 25 F_6 families indicated that C_2H_2-reduction and H_2-evolution characteristics generally changed in a uniform manner between 28 and 35 d after imbibition (Fig. 1). All but one family had higher rates of C_2H_2 reduction and H_2 evolution on d 35 than on d 28. Significant differences

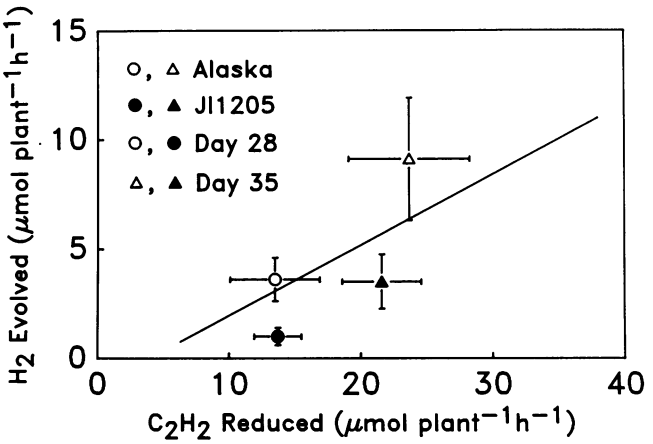

Figure 1. Regression analysis of apparent nitrogenase activity (C_2H_2 reduction) and H_2 evolution from *R. leguminosarum* 128C53 in pea root nodules of 25 F_2-derived F_6 families from a cross between Alaska and JI1205. The H_2 evolution measured is the H_2 released from nitrogenase less any H_2 oxidation by uptake hydrogenase. Mean values of six plants from each of the 25 families 28 and 35 d after germination are represented by the line $Y = 0.32X - 1.27$ ($R^2 = 0.64$; $P \leq 0.001$), which was obtained by regression analysis. Mean values ± SE for Alaska (O, Δ) and JI1205 (●, ▲) controls on d 28 (O, ●) and d 35 (Δ, ▲) are indicated separately.

in RE values of individual families were not detected over the period tested. RE values on d 28 and 35 for the 25 F_6 families were well correlated ($r = 0.75$; $P \leq 0.001$), and RE phenotypes assigned to each family relative to JI1205 and Alaska controls also were strongly correlated between the two assay dates ($r = 0.85$; $P \leq 0.001$). On the basis of those data, it was concluded that RE phenotypes in F_6 families could be assessed accurately between d 28 and 35.

When RE values of six individual plants in each of 173 F_2-derived F_6 families were assayed 33, 34, or 35 d after imbibition, 41 families were identified as containing potential RE segregants. Shoots of possible segregants were rerooted under hydroponic conditions in the +N nutrient solution, and at least one seed was recovered from each plant. In a few cases, more than six seeds were produced, and six F_7 progenies from each F_6 plant were re-tested for RE phenotypes. In most cases, fewer than six seeds were recovered, so one F_7 plant was grown from each F_6-derived line to produce F_8 seed. RE phenotypes of F_7 and/or F_8 progenies indicated that only 8 of the original 173 F_2-derived F_6 families had segregated for that trait. Homozygous lines breeding true for Hup^{++} and Hup^{+} phenotypes were identified from progenies of those eight pairs of isolines by repeated tests for RE and $^3H_2$ incorporation.

Qualitative Genetic Analyses

$^3H_2$-incorporation tests of 165 F_2-derived F_6 families that were not segregating for RE showed a wide range of Hup activities (Fig. 2a). The 165 families were divided into 90 JI1205 and 75 Alaska Hup phenotypes, relative to the mean values of JI1205 and Alaska controls across all assays, by the following criteria: mean Alaska Hup ± (SE) (t_α) = Alaska phenotype; mean JI1205 Hup ± (SE) (t_α) = JI1205 phenotype. Five plants with Hup activities between those two distributions were assigned to the closer distribution (Fig. 2b).

The possibility that the 90:75 segregation pattern in F_6 families represented a 1:1 ratio associated with segregation of a single genetic locus was supported by analyses of the F_1 population and by testcrosses between JI1205 and F_1 plants (Table II). The ostensible segregation of the F_1 population was based on two plants with $^3H_2$-incorporation activities of 29.3 and 30.6 nmol H_2 mg nodule^{-1} h^{-1}, which were below the midpoint between the two parental controls but still markedly greater than the Hup^{+} Alaska control (mean ± SE: 21 ± 1.3). Similar $^3H_2$-incorporation activities at the lower end of the distribution of Hup^{++} phenotypes in progenies from the JI1205 × F_1 and F_1 × JI1205 backcrosses accounted for the four unpredicted Hup^{+} plants.

Characteristics of Hup^{++}/Hup^{+} Pea Isolines

Direct tests of $^3H_2$-incorporation confirmed that the eight pairs of lines segregating for RE with *R. leguminosarum* 128C53 in the F_6 generation also produced significantly different Hup activities in that strain (Table III). Although not all Hup^{++}/Hup^{+} pea isolines altered Hup activity significantly in two other rhizobial strains, 128C30 and 175R1, 9 of the 16 comparisons showed significant differences in Hup activity parallel to the effects observed on 128C53. Two pairs (35/128C30 and 37/175R1) showed a significant reversal of those effects (Table III).

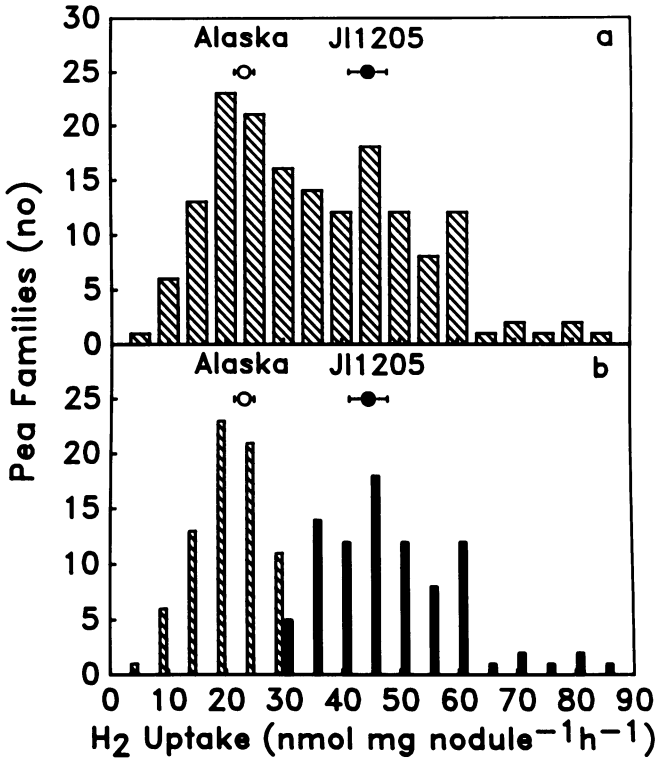

Figure 2. Hup activity of *R. leguminosarum* 128C53 in symbioses with 165 F_2-derived F_6 families from a cross between Alaska and JI1205. Hup activities are shown as the distribution of (a) all families and (b) separate Hup^{++} (solid bar) and Hup^{+} (cross-hatched bar) phenotypes determined relative to the parental controls. Two families with values of 99 and 110 nmol H_2·mg nodule^{-1}·h^{-1} are not shown.

Table II. *Segregation of Hup Phenotypes Produced in R. leguminosarum 128C53 by Pea Lines Originating from Crosses between the Cultivars JI1205 and Alaska*

Predicted segregations test a single-gene model, and probabilities are based on χ^2 tests.

Population	Observed Segregation	Predicted Segregation	P for Expected Ratio
	Hup^{++}:Hup^{+}	Hup^{++}:Hup^{+}	
	no. of families or plants		
F_2-derived F_6 families	90:75	82.5:82.5	(0.50–0.20)
F_1	33:2	35:0	(0.75–0.50)
F_1 × Alaska[a]			
Alaska × F_1	14:28	21:21	(0.02–0.01)
F_1 × JI1205[a]			
JI1205 × F_1	38:4	42:0	(0.75–0.50)

[a] Observed ratios are totals from reciprocal crosses.

When the eight pairs of pea isolines were grown on 8 mM NH_4NO_3 to assess phenotypic differences without rhizobial dependence, only three had similar flowering dates with no significant differences in dry weight or N assimiation (Table IV). Those pairs, 17, 83, and 117, (Hup^{++} lines: 17-2, 83-4,

Table III. *Hup Activity of Three Strains of R. leguminosarum in Symbioses with Eight Pairs of Nearly Isogenic Pea Lines Produced from F_2-derived F_6 Families Segregating for Hup^{++} and Hup^{+} Phenotypes*

The original parents JI1205 and Alaska are given as controls. Values representing the mean of three replicate plants were compared by a *t* test within each pair of isolines for host genotype effects.

Pea Line	*Rhizobium* strain		
	128C53	128C30	175R1
	nmol H_2·mg nodule^{-1}·h^{-1}		
JI1205 (parent)	35.0**	26.7**	13.5*
Alaska (parent)	25.2	7.0	4.1
17-2	25.0*	23.6*	10.4
17-4	17.8	10.1	9.8
35-1	42.2*	49.5*	28.7*
35-5	50.5	35.5	39.6
37-2	31.3*	60.9**	10.6**
37-4	21.6	18.1	17.6
83-4	20.5***	23.0	19.9**
83-5	15.7	18.1	8.5
117-4	21.8***	21.8***	23.6***
117-6	12.2	7.8	6.0
118-1	26.5***	17.1*	12.2*
118-4	17.8	10.1	7.5
119-2	23.2***	13.2***	8.3
119-5	13.5	7.2	7.8
133-5	14.7**	11.6	6.0
133-6	10.1	9.8	9.3

*,**,*** Pea host effect significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

Table IV. *Characteristics of Pea Lines Identified in Table III*

Growth data are reported as means for six replicate plants after 35 d on 8 mM NH_4NO_3. In pea lines that failed to flower by d 35, one representative plant was grown until flowering.

Pea line	Traits		Growth		
	Tendrils	Age at flowering	Plant height	Total dry weight	Total N content
		d	*cm*	*g·plant^{-1}*	*mg·plant^{-1}*
JI1205 (parent)	–	48	36	1.70	86
Alaska (parent)	+	25	90	1.81	90
17-2	+	31	107	2.32	120
17-4	+	31	112	2.71	128
35-1	+	39	18	0.92	47*
35-5	+	42	22	1.18	67
37-2	–	44	18	1.30**	71*
37-4	+	29	109	1.92	95
83-4	+	30	110	2.36	104
83-5	+	30	114	2.60	113
117-4	+	37	76	1.97	91
117-6	+	37	64	1.61	80
118-1	–	54	64	1.61**	78***
118-4	–	57	18	0.88	45
119-2	+	29	118	2.96**	140***
119-5	+	29	104	2.31	100
133-5	+	57	23	1.23	61
133-6	–	44	23	1.06	61

*,**,*** Differences between paired lines significant at $P \leq 0.05$, 0.01, or 0.001, respectively.

117-4; Hup^{+} lines: 17-4, 83-5, 117-6) were thus identified as being most suitable for physiological studies.

Effects of Hup^{++}/Hup^{+} Pea Isolines on N_2 Fixation

Symbiotic tests of the three most similar pairs of Hup^{++}/Hup^{+} pea lines in associations with isogenic rhizobial strains 518 (Hup^{+}), 523 (Hup^{-}), and 524 (Hup^{-}) showed no general pattern of greater N_2 fixation associated with either Hup^{+} rhizobia relative to isogenic Hup^{-} strains or Hup^{++} peas relative to Hup^{+} pea isolines. In two out of three comparisons between Hup^{++} and Hup^{+} pea isolines inoculated with the Hup^{+} strain 518, the Hup^{+} isoline contained significantly more N_2-derived N (Table V). In 3 of 12 comparisons between Hup^{+} and Hup^{-} isogenic strains in the same pea line, the Hup^{-} strain fixed significantly more N_2, while in 1 of the 12 comparisons the Hup^{+} rhizobial strain fixed significantly more N_2.

A dominant genotypic effect of the plant, as opposed to the rhizobia, was evident when results were averaged across pea lines and/or bacterial strains (Table VI). Thus, the Hup^{+} plants produced 8.2% more dry matter ($P \leq 0.05$) and fixed 12.6% more N_2 ($P \leq 0.05$) than the Hup^{++} lines across all rhizobial strains, while there were no significant differences in N_2 fixation by the isogenic rhizobial strains when averaged across all plant lines. There were no significant differences in the initial seed mass or seed N content between paired lines. The average seed weighed 294 mg and contained 10.7 mg N at planting.

DISCUSSION

Although there are many known cases in which two host plant species produce different levels of Hup activity in rhizobial symbionts (10, 12, 19, 21, 23), such materials cannot be used in physiological studies to clarify effects of Hup activity on the intact symbiosis because they differ in many other traits. Three of the eight Hup^{++}/Hup^{+} pea isolines described in this report (Tables III–V) overcome that problem to a greater extent than any other plant materials presently available. Results suggest that these pea isolines were produced by segregation of a single plant gene in the F_6 generation (Table II; Fig. 2). Most of the eight pairs of pea lines produce similar changes in Hup activity of two other *Rhizobium leguminosarum* strains (Table III), so the physiological differences affecting Hup probably have significance in *Pisum sativum* L. beyond the *Rhizobium*-pea materials studied here. At this time, however, no data are available to indicate the physiological basis of the different Hup phenotypes in these isolines. Presumably, the plant effects are mediated by quantitative or qualitative differences in compounds transported into the peribacteroid space. Differences in salt concentration, for example, might affect rhizobial membrane potential and

Table V. *Growth Under N_2-Dependent Conditions for Three Pairs of Nearly Isogenic Pea Lines Producing Hup^{++} or Hup$^+$ Phenotypes in Hup$^+$ Rhizobia*

Plants were inoculated with Hup$^+$ rhizobial strain 518 or a related Hup$^-$ strain produced by Tn*5:mob* mutagenesis. Peas were harvested after 56 (families 17 and 83) or 63 (family 117) d of growth, when two pods were filled. N_2 fixed was calculated as (total plant N at harvest) − (original seed N). Values are means of four replicates with four plants in each.

Pea Line	Pea Phenotype[a]	Rhizobial Symbiont	Rhizobial Phenotype[b]	Shoot Mass	Root Mass	Total Dry Weight	Total N_2 Fixed
					g·plant^{-1}		*mg·plant^{-1}*
17-2	Hup^{++}	518	Hup$^+$	4.41	0.35	4.76	112
17-2	Hup^{++}	523	Hup$^-$	5.11	0.46	5.57	147
17-2	Hup^{++}	524	Hup$^-$	4.91	0.41	5.32	138
17-4	Hup$^+$	518	Hup$^+$	5.05	0.40	5.45	148
17-4	Hup$^+$	523	Hup$^-$	5.02	0.41	5.43	144
17-4	Hup$^+$	524	Hup$^-$	5.09	0.41	5.50	148
83-4	Hup^{++}	518	Hup$^+$	4.51	0.43	4.94	126
83-4	Hup^{++}	523	Hup$^-$	3.92	0.35	4.27	108
83-4	Hup^{++}	524	Hup$^-$	4.61	0.42	5.03	116
83-5	Hup$^+$	518	Hup$^+$	4.61	0.38	4.99	122
83-5	Hup$^+$	523	Hup$^-$	4.45	0.41	4.86	125
83-5	Hup$^+$	524	Hup$^-$	4.51	0.46	4.97	122
117-4	Hup^{++}	518	Hup$^+$	6.03	0.56	6.59	158
117-4	Hup^{++}	523	Hup$^-$	5.52	0.51	6.03	147
117-4	Hup^{++}	524	Hup$^-$	6.22	0.65	6.87	156
117-6	Hup$^+$	518	Hup$^+$	6.88	0.53	7.41	180
117-6	Hup$^+$	523	Hup$^-$	6.45	0.51	6.96	175
117-6	Hup$^+$	524	Hup$^-$	7.25	0.63	7.88	194
			$LSD_{0.05}$	0.41	0.06	0.43	12

[a] When assessed with *R. leguminosarum* 128C53. [b] When assayed on Alaska pea (9).

thereby alter Hup activity (18). Differences in other compounds might produce more specific effects on root nodule bacteroids.

Two lines of evidence suggest that the Hup^{++} and Hup$^+$ phenotypes of JI1205 and Alaska examined in this study resulted primarily from a difference at a single genetic locus. One line of evidence is based on the X^2 values of Hup^{++}:Hup$^+$ segregation ratios observed in the F_2-derived F_6 families, the F_1 population, and the backcrosses between JI1205 and the F_1 plants (Table II). Those values provided moderately good support for the single-gene model, but segregants in the backcrosses between Alaska and the F_1 plants did not confirm the hypothesis. The small number of plants analyzed and the nature of the phenotypic assay may account for the latter difficulty. Additional evidence supporting a single-gene model is based on the number of isolines recovered in the F_6 families. If one major locus influenced host plant control of rhizobial Hup phenotypes, then 10.8 of the 173 F_6 families analyzed should have segregated for Hup^{++} and Hup$^+$ phenotypes during their production on F_5 plants (1). On the basis of that prediction, a reasonable recovery rate of 8 out of 10.8 was observed. This variation from the expected number of segregating isolines may reflect the small number of families tested, or it may be related to the fact that the indirect RE measurement, rather than the direct, but more expensive and hazardous, $^3H_2$-incorporation assay, was used to assess Hup phenotypes during the F_6 analyses.

Assaying Hup phenotypes in legumes is a technically challenging task. First, the plants lack the capacity to metabolize H_2, so all Hup phenotypes reflect a host plant effect on a microbial symbiont that contains the Hup system. Second, because water is the ultimate produce of H_2 uptake, Hup assays must be either very brief or indirect. The small size and gaseous nature of the H_2 molecule are other factors whose effects can be minimized by using short assays. Thus, all determinations of Hup phenotypes in this study were based on short-term measures of enzymatic activity in bacteria which are genetically and physiologically separate from the plants that form the basis of the genetic study. It is not surprising, therefore, that even though the original parents were presumed to be homozygous, the F_1 population showed an illusive nonuniformity in Hup phenotypes (Table II). It is likewise understandable that difficulties associated with the assays may have affected Hup segregation ratios in other populations.

The Hup^{++}/Hup$^+$ pea isolines developed in this study are closely related by traditional plant breeding criteria (1), but they differ greatly at the molecular level. Thus, variations within paired lines under nonsymbiotic conditions (Table IV) show that major differences in growth and development can exist in such similar materials. Those differences may reflect segregation of other genes in the F_6 generation, such as internode length in families 37 and 118, or they may indicate pleiotropic effects of the gene controlling Hup phenotype.

Table VI. *Plant and Rhizobial Effects on Growth and N_2 Fixation Results Reported in Table V*

Plant Factor	Bacterial Factor	Total Dry Weight	Total N_2 Fixed
		$g \cdot plant^{-1}$	$mg \cdot plant^{-1}$
Hup^{++} lines[a]	518 (Hup^+)[b]	5.43	132
	523 (Hup^-)	5.29	134
	524 (Hup^-)	5.74	137
Hup^+ lines	518 (Hup^+)	5.95	150
	523 (Hup^-)	5.75	148
	524 (Hup^-)	6.12	155
	$LSD_{0.05}$	0.22	10
Hup^{++} lines	All strains	5.49	134
Hup^+ lines	All strains	5.94	151
	$LSD_{0.05}$	0.35	15
All lines	518 (Hup^+)	5.69	141
	523 (Hup^-)	5.52	141
	524 (Hup^-)	5.93	146
	$LSD_{0.05}$	0.43	19

[a] Phenotype when assessed with *R. leguminosarum* 128C53. [b] Phenotype when assayed on Alaska pea (9).

There was no apparent link between internode length and Hup phenotype, because several other F_6 families that segregated into tall and short plants on d 35 showed no differences in Hup activity.

Although Hup^+ rhizobia have potential physiological advantages over Hup^- strains (10, 29), actual increases in N_2 fixation or plant growth have been difficult to document. In the *Bradyrhizobium*-soybean symbiosis, data from comparisons of a single isogenic pair of Hup^+ and Hup^- strains have been interpreted as showing both positive and negative effects of the Hup^+ phenotype on N_2 fixation (11, 14, 17). In *Rhizobium*-pea symbioses, no clear benefit of Hup^+ strains relative to Hup^- rhizobia has been evident (9, 30, 33). In fact, data indicate that other, unknown genetic factors in *R. leguminosarum* are more important than the Hup^+ phenotype (9, 31). Given that background, the various differences in N_2 fixation by isogenic Hup^+ and Hup^- rhizobia in six pea lines (Table V) were not surprising. Other workers have suggested that Hup^- rhizobia may fix more N_2 than Hup^+ strains if O_2 oxidation by Hup activity decreases an already limiting O_2 concentration in the root nodule (11). If O_2 concentrations differ in nodules of various pea isolines, one would expect the relative amounts of N_2 fixation by Hup^+ and Hup^- rhizobia to be altered.

Physiological traits associated with the Hup^{++} phenotype in pea line 117-4 apparently impaired symbiotic performance of both Hup^+ and Hup^- rhizobia (Table V). Indeed, when results were averaged across all bacterial strains, the Hup^{++} peas produced less dry matter and fixed less N_2 than Hup^+ lines (Table VI). Such results cannot be attributed to inherently poor growth of the Hup^{++} lines because growth and N assimilation were not significantly different in the Hup^{++}/Hup^+ pea families 17, 83, or 117 on NH_4NO_3 (Table IV). Neither can a negative effect of the Hup^{++} phenotype be discounted by claiming that the amount of N_2 fixed during the experiment failed to allow the expression of Hup benefits. In these studies, the 153.2 mg N harvested in the average plant was derived from 10.7 mg N in the original seed and 142.5 mg N_2 fixed. Thus, N_2 fixation produced 3.84 doublings in plant N. Postulating a geometric model in which any effect of Hup activity was expressed equally through each doubling and using the $LSD_{0.05} = 12$, allows one to calculate that any Hup effect producing a 4.1% change in N_2 fixation rate would have been detected as being significant in the experiment reported in Table V.

Although the Hup^{++} lines used for the experiment reported in Table V were later discovered to be still segregating 3:1 for Hup^{++}:Hup^+ phenotypes, that fact favors, rather than discredits, the conclusion that Hup^{++} plants fix less N_2 than the Hup^+ isolines. If the Hup^{++} phenotype really were physiologically superior to the Hup^+ condition, the presence of any Hup^+ plants could not have decreased N_2 fixation significantly below that of the Hup^+ control. If the Hup^+ phenotype were superior to the Hup^{++} condition (as was actually observed), then the inclusion of the Hup^+ segregant with the Hup^{++} plants would increase N_2 fixed and make differences between isolines even more difficult to detect. Thus, the detection of a significant difference in the present case (Table VI) strongly supports the conclusion that Hup^+ plants induce more N_2 fixation that Hup^{++} isolines. Additional progeny tests run after the experiment reported in Table V identified homozygous Hup^{++} lines in families 17, 83, and 117, which are now available to other investigators.

The biological materials and experimental results reported here open potential new avenues of investigation and emphasize that caution must be exercised in drawing conclusions about the practical significance of rhizobial Hup activity. The nearly isogenic Hup^{++}/Hup^+ pea lines 17, 83, and 117 offer new tools for studying how different levels of Hup activity interact with other physiological processes in root nodules, and they may help identify transmissible shoot factors that influence Hup activity in peas (4). Until it is known how the

multiple factors involved in transfer of electrons from H_2 to O_2 interact with electron transport in normal respiratory pathways and in those specifically associated with nitrogenase, a general conclusion about the benefit or disadvantages of H_2 uptake is premature. A few appropriate genotypes of both rhizobia and legumes are now available to begin such studies.

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