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Hybrid Weakness in *Phaseolus vulgaris* L. II. Disruption of Root-Shoot Integration

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Abstract. We have been examining the importance of the root system on shoot growth and development using a developmentally disabled hybrid of the common bean *Phaseolus vulgaris* L. Parental cultivars (*P. Vulgaris* cv. Redkloud of Mesoamerican origin, and *P. vulgaris* cv. Batt of Andean origin) grow normally, but crosses produce F1 hybrids exhibiting hybrid weakness associated with reduced root and shoot growth. In this study, applications of benzylaminopurine (BAP) to roots of F1 hybrids increased the number of root tips and leaves. Reciprocal grafting was used to study the effects of the root system on shoots. Grafting of roots of the Mesoamerican cultivar onto shoots of F1 hybrids increased the cytokinin concentrations in leaves of F1 hybrids and removed the characteristics associated with hybrid weakness. To determine whether factors in the xylem sap enhanced leaf growth, leaf discs were incubated on sap collected from Mesoamerican and Andean cultivars. Sap from Mesoamerican plants enhanced the growth of leaf discs excised from F1 hybrids more than sap collected from Andean cultivars. Estimates of the transport of zeatin riboside (ZR)–type cytokinins from roots of F1 hybrids indicated that transport out of hybrid roots was reduced compared with those transported out of Mesoamerican or Andean roots. Results suggest that ZR-type cytokinins are involved in hormonal integration between roots and shoots of *P. vulgaris* and that one of the barriers to hybridization between Andean and Mesoamerican landraces is related to hormone transport.

Key Words. *Phaseolus vulgaris*—Cytokinin—Grafting—Root to shoot communication—DL genes

Our understanding of how roots and shoots are integrated remains unclear. Several investigators have used various approaches to demonstrate how the root system is limiting to shoot growth and development. White and Castillo (1989) used a grafting technique to study the effects of root genotype on yield in *Phaseolus vulgaris*. Trejo and Davies (1991) looked for evidence of root-to-leaf signals under soil drying in *Phaseolus*. To study the role of roots in shoot responses, we used a genetic technique using the DL (dosage lethal) system of *P. vulgaris* described by Shii et al. (1980,1981) and others (Gepts and Bliss 1985, Singh and Gutierrez 1984). In *Phaseolus*, F1 crosses between specific cultivars (Mesoamerican cultivar [Redkloud] and Andean cultivar [Batt]) of *P. vulgaris* yield F1 plants that exhibit lethal symptoms at the seedling stage and fail to grow to maturity. Shii et al. (1980) described the genetics of this system based on the results of a backcrossing experiment. Their results indicate that (1) the phenotype is controlled by at least two genes, (2) the incompatibility is due to nuclear and not cytoplasmic effects, and (3) lethality increases with increasing dosage. In addition to describing the genetics of this system, they investigated the factors responsible for the hybrid lethality. They found that most of the seedlings were developmentally dysfunctional, especially when grown at higher temperatures (35°C vs 28°C). Symptoms included decreased leaf and root growth, leaf chlorosis, the development of adventitious roots on the stems, and eventual death. This lethality could be avoided by culturing hybrids in the presence of benzylaminopurine (BAP) or by grafting the root system of the Mesoamerican cultivar onto the F1 hybrid shoots at an early stage in development. There was no rescue effect, however, when roots of the Andean cultivar were grafted onto F1 hybrid shoots. The results of these studies suggest that the DL system is characterized by independent root and shoot effects and that a cytokinin deficiency may be an important component of hybrid lethality in this system. This system is ideal, therefore, to study the integration of

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Abbreviations: BAP, benzylaminopurine; DHZ, di-hydrozeatin; DL, dosage lethal; ZR, zeatin riboside.

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roots and shoots with respect to cytokinins. In a companion article (this issue) we have found that although cytokinins are reduced in roots and leaves of the F1 hybrids, they are high in stems, suggesting that a breakdown occurs in the transport or allocation of cytokinins instead of a deficiency of cytokinins (Reiber and Neuman 1999).

The role of cytokinins in root-to-shoot integration has been considered by a number of investigators with ambiguous results. Cytokinins are plant hormones involved in numerous physiologic events related to growth and development (Incoll and Jewer 1987). Although approximately 30 cytokinins (mostly conjugates) have been identified in higher plants, the biologic function of these compounds, their location within cells and tissues, and the enzymes and genes involved in their regulation are not clearly understood. In some studies, cytokinins appear to have a role in the integration between roots and shoots. For example, Dieleman et al. (1998) found that some root stocks affecting physiologic responses in shoots are related to cytokinin concentration, presumably produced in the roots. Clearly, the effects of root-sourced cytokinins on shoots is complex and probably also involves developmental receptiveness (Vondrakova et al. 1998). Although the pool of biologically active cytokinins is related to a number of processes, including biosynthesis, mobilization of storage forms, deactivation by conjugation, and sidechain removal (Laloue and Pethe 1982), in this study we have focused on zeatin riboside (ZR), the cytokinin most associated with transport between roots and leaves. In addition to the presence of ZR in xylem sap, applications of ZR have been shown to enhance growth of leaf discs in some systems (Neuman et al. 1990). These data suggest a role for ZR as a messenger between roots and shoots.

To better understand the mechanisms regulating the integration between roots and shoots, we investigated changes in the concentrations of ZR in two cultivars of *P. vulgaris* (cv. Redkloud and cv. Batt) and F1 crosses carrying the DL phenotypes. Crosses between Redkloud and Batt are characterized by F1 hybrids that demonstrate traits as described by Shii et al. (1980, 1981), including stunted root and shoot growth, senescing leaves, and adventitious root development on stems. On the basis of our studies and those reported in the literature we hypothesized that altered ZR transport from roots to shoots could influence growth and development in crosses between *Phaseolus* of Mesoamerican and Andean origin. In this study, estimates of the export of ZR in xylem sap collected from roots under pressure were determined. In addition, we studied the leaf growth-promoting properties of the xylem sap collected from parental cultivars and F1 hybrids. Finally, we examined whether rootstocks from parents could mediate shoot growth of the F1 hybrids and alter ZR concentrations in tissues.

Materials and Methods

Plant Growth and Grafting

Seedlings of bean (*P. vulgaris* cv. Redkloud; *P. vulgaris* cv. Batt; *P. vulgaris* cv. Redkloud × cv. Batt; *P. vulgaris* cv. Batt × cv. Redkloud) were grown from seeds in a controlled room at 30–35°C for a 16-h photoperiod, 300 μE $m^{-2}s^{-1}$. For grafting, shoot and root unions were interchanged among 6–7-day-old plants by cleft grafts wrapped in Parafilm (American National Can Co, Greenwich, CT) after White and Castillo (1987). After unions were made, plants were enclosed in clear plastic bags until graft unions were formed. Growth was monitored by counting the number of leaves produced after 4 weeks.

Hormone analysis

To assess the effects of interchangeable plant parts on hormone concentrations, roots and leaves were collected for hormone analysis from graft combinations after 4 weeks of postgraft growth. Plant material was harvested and frozen in liquid nitrogen and then stored at −70°C. Frozen material was ground in liquid nitrogen, extracted in ice-cold HPLC grade methanol buffer, shaken for 30 min, and centrifuged at 2,500 rpm for 10 min. Supernatant was evaporated, dried, and partially purified as previously described (Neuman et al. 1990). Extracts were dried and resuspended in 0.01 M phosphate buffer (pH 7.4).

For hormone analysis, ZR concentrations in tissues were measured in young growing leaves and xylem sap as previously described (Neuman et al. 1990). The method and cross-reactivities of the antisera for ZR are previously reported (Smit et al. 1990). Because there are appreciable cross reactivities of the ZR antiserum with di-hydrozeatin (DHZ, 14.1%) and zeatin (Z, 7.6%) , cross-reactants could contribute to the total estimated concentration of the cytokinin of interest. [3H]-DHZ (approximately 2,500 dpm, 30-60 Ci/mmol; Amersham, UK) was added to all samples to check losses by isotope recovery. Recoveries varied between 40–80% for tissues and close to 90% for xylem sap. Although samples were corrected for recovery, no corrections were made for added [3H]-DHZ because the ratio of tritium-labeled DHZ to endogenous concentrations within plant tissues was small and much less than the sensitivity of the assay (0.01 pmol).

For xylem sap analysis, roots were washed free of planting media, excised from the plants, placed in a vessel filled with 0.1 strength Hoagland's solution and pressurized to 0.3 MPa (Smit et al. 1990). One to 2 mL of xylem sap was collected from excised root systems while the rate of sap flow was determined gravimetrically (g min^{-1}). ZR cytokinin concentrations in xylem sap collected from roots were estimated by immunoassay after partial purification (Smit et al. 1990). [3H]-DHZ (Amersham, UK) was added to all samples to check losses by isotope recovery as described previously.

Hormone fluxes were derived from the mass flow of sap (g min^{-1}) from pressurized roots and from measurements of concentrations of ZR cytokinins. The sap flow at 0.3 MPa applied pressure was assumed to be representative of sap flow *in vivo* driven by a 0.3-MPa water potential difference from the roots to the leaves. A water potential difference of this magnitude from roots to leaves is within a normal daily range for these cultivars (data not shown).

To determine the effects of exogenous applications of cytokinins on F1 hybrids, hybrids were grown in aerated hydroponic cultures (0.1 strength Hoagland's) in the presence of N^6-benzyladenine (Sigma, St. Louis, MO) at concentrations of 0, 0.05, 0.25, 0.5, 2.5 and 5.0 μM.

Table 1. Cytokinin concentrations (zeatin riboside equivalents) in roots and leaves of root-shoot graft combinations between Batt and Redkloud cultivars of *Phaseolus vulgaris* and their F1 hybrids.

Graft combination (root/shoot)	"ZR" (pmol g^{-1}) Root	Leaf	No. of leaves after 4 wk
Batt/Redkloud[a]	32.6 ± 7.4	10.9 ± 2.9	2.1 ± 1.2
Redkloud/Batt	49.8 ± 4.9	80.4 ± 7.9	6.3 ± 2.2
Redkloud/F1 hybrid	67.5 ± 9.3	106.8 ± 6.2	5.1 ± 2.0
Batt/F1 hybrid	11.7 ± 3.9	12.9 ± 4.8	1.3 ± 1.0
Redkloud/Redkloud	56.1 ± 12.2	143.7 ± 21.8	8.0 ± 2.0
Batt/Batt	45.2 ± 3.2	61.1 ± 4.9	6.6 ± 1.0

[a] Batt/Redkloud: root from Batt; shoot from Redkloud.
Means followed by SE. ($n = 3$–5 plants)

Four plants from each genotype were harvested after 4 weeks, and dry weights of roots and shoots were determined. Root growth was estimated by counting the numbers of root tips each week.

In Vitro Leaf Growth Assays

To study the effect of possible growth-enhancing or growth-inhibiting substances in xylem sap, leaf discs were excised from growing leaves and incubated in xylem sap collected from Mesoamerican and Andean parents. Xylem sap was collected as described above for hormone analyses, lyophilized, and resuspended at 2× concentration. No difference was found in pH or solute concentration between sap collected from Batt or Redkloud parents (data not shown). For the *in vitro* assay, discs were excised from growing leaves, incubated on solutions of sap, and the diameter measured after 24 h as a control. The *in vitro* growth response to 10 mM KCl plus 10 mM sucrose was also determined. All leaf disc assays were performed as previously described (Smit et al. 1990).

Analysis and Statistics

Data were analyzed using *t* test. Each experiment was repeated at least twice. Results are pooled from several experiments and are considered statistically significant at $p < 0.05$ or 0.01. Comparisons between means were evaluated by *t* test at $p = 0.01$ and 0.05 level of error (Sigma Stat 2.0, Jandel Sci.).

Results

Results of the grafting study indicate that Redkloud root systems, but not Batt root systems, restored normal shoot growth to F1 hybrids (Table 1). Self grafts between Redkloud and Batt produced plants with normal leaf growth (6–8 leaves). Grafts of Batt roots to Redkloud shoots produced plants that grew poorly, whereas grafts of Redkloud roots to Batt shoots produce plants with leaf numbers comparable to the parental cultivars. When unions were made between roots of Redkloud and shoots of young F1 hybrids, normal growth was restored to the F1 shoots, whereas unions between roots of Batt and shoots of F1 hybrids did not produce plants with the normal complement of leaves.

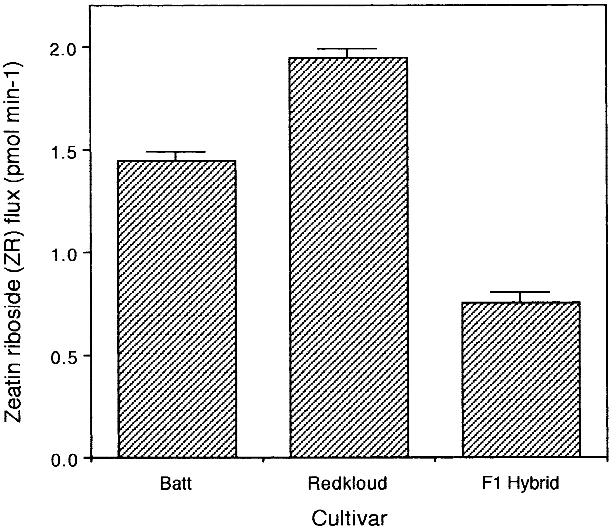

Fig. 1. Cytokinin transport in Redkloud and Batt cultivars of *Phaseolus vulgaris* and their F1 hybrids. Data are derived from estimates of the mass flow of sap collected under pressure and immunologic measurements of ZR.

Measurements of the cytokinin concentrations of roots and leaves of the graft combinations indicate that the highest concentrations of cytokinins were found in Redkloud/Redkloud unions (Table 1). Lower concentrations were found in roots and leaves of the Batt/Batt grafts. In unions in which roots of Redkloud were grafted to shoots of F1 hybrids, cytokinin concentrations approached that of the Redkloud control. Unions between Batt roots and F1 shoots produced plants with greatly reduced concentrations of cytokinins in roots and leaves.

Estimates of ZR-type cytokinins in the xylem sap of parental and F1 hybrids indicate the concentration of ZR was greatly reduced in sap of F1 hybrids compared with either of the parental cultivars (Fig. 1). Estimations of the putative delivery rate of cytokinins out of roots may be determined by multiplying the root flux rate (mL min^{-1}) by the hormone concentration (pm mL^{-1}) to obtain a delivery rate (pm min^{-1}). With this approach the data indicate the transport of cytokinins out of roots of F1 hybrids was greatly reduced compared with that of the parents.

A feeding experiment was used to test the effects of hormone replacement on growth of F1 hybrids. Exogenous applications of BAP fed to the roots of F1 hybrids restored normal root growth in a dose-dependent manner (Table 2). There was no effect of exogenous applications of BAP on growth and development of either parent (data not shown).

Table 2. Effects of exogenous application of BAP on dry mass accumulation and number of root tips of F1 hybrids of *Phaseolus vulgaris* after 4 wk.

Cytokinin treatment BAP (μM)	No. of root tips	Mass (g)
0	77 ± 18	1.8 ± 0.2
0.05	91 ± 22	1.7 ± 0.8
0.25	152 ± 20	2.6 ± 1.2
0.5	389 ± 59	4.4 ± 3.6
2.5	700 ± 121	7.6 ± 2.6
5.0	659 ± 83	8.9 ± 2.1

Means followed by SE. ($n = 5$–6 plants)

An *in vitro* leaf disc growth assay was performed to determine whether sap contained constituents that can influence leaf growth (Table 3). 10 mM KCl plus 10 mM sucrose were used as controls in this experiment. KCl and sucrose sustained growth of discs excised from Batt and Redkloud. Discs collected from leaves of F1 hybrids did not expand as much as discs collected from either of the parents in the control media. Sap collected from Batt supported the growth of discs excised from Batt plants, whereas discs collected from the Redkloud cultivar grew less in sap collected from Batt plants than when incubated in its own sap. Interestingly, discs from the Batt cultivar grew more on sap from Redkloud than on their own sap. Discs excised from the F1 hybrids grew most on sap collected from the Redkloud parent compared with growth when discs were incubated on sap from Batt plants or KCl plus sucrose. 10^{-5}M ZR enhanced growth of leaf discs of F1 hybrids. There was no effect on discs excised from Batt or Redkloud (Table 3).

Discussion

At a cellular level, cytokinin concentrations in cells and organs depend on various factors, including biosynthesis and/or uptake, metabolic interconversion, inactivation, and degradation. How cytokinin concentrations are controlled at the organ level is poorly understood. To begin to understand events leading from the perception of a hormonal change to the manifestation of a response, an understanding of whole plant integration is needed. Analysis of the concentrations of cytokinins in organs provides one means of investigating cytokinin signaling at a whole plant level of organization. However, this problem is confounded by the fact that there are approximately 30 cytokinins with various roles, most of which are poorly understood. Conclusive identification of which transported cytokinin is most important has yet to be achieved, but ZR has been suggested to be an integrator between roots and leaves because of its presence in xylem sap (Incoll et al. 1990, Lujeune et al. 1994, Neuman et al. 1990). In addition, although several authors have suggested that root-produced cytokinins are important in shoot responses, there is at least one reported suggestion that concentrations of cytokinins in the xylem sap of the roots is determined by the shoot (Beveridge et al. 1997). This study is focused on the idea that root-produced cytokinins may play a role as a limiting factor in developmental shoot responses in plants. Although this report focuses on a single cytokinin (ZR), it is likely that plant growth and development is controlled by synergistic effects, whereby combinations of hormones such as auxins and cytokinins act together.

In this study, grafting was used to separate root and shoot characteristics (Hussein and McFarland 1994, Izquierdao and Hosfield 1982, Sanders and Markhart 1992, Shii et al. 1981, White and Castillo 1989). Reciprocal grafts and grafts between F1 hybrids and parental cultivars allowed separation of root and shoot effects on the growth of the F1 hybrids. The findings of this study confirm observations made by Shii et al. (1980, 1981) that cytokinins are involved in the growth responses of bean landraces characterized by hybrid weakness.

Why graft unions between F1 hybrid shoots and Mesoamerican (Redkloud) roots were more successful than unions between F1 hybrid shoots and Andean (Batt) roots is unclear. One possibility may be slight differences in patterns of organ growth between Andean and Mesoamerican landraces. In a companion article (this issue), we found that the early rate of biomass accumulation in roots was greater in Redkloud than in Batt plants, although final biomass accumulation at 4 weeks was the same (Reiber and Neuman 1999). It is possible, therefore, that crosses between Andean and Mesoamerican landraces do not hybridize well because of differences in developmental timing in the formation of roots. Rieseberg and Ellstrand (1993) suggested that first-generation hybrids are a mosaic of parental and intermediate physiology rather than solely intermediate characteristics (Alston 1965, Fahselt and Ownbey 1968). Developmental instability has been reported in a number of hybrids (Levin 1970, McDade 1990, Rieseberg and Ellstrand 1993, Wagner 1962). One explanation that has been put forth to account for reduced developmental stability in hybrids has been alterations in chemical compounds in hybrids where both parents grow normally. In some cases, a considerable number of parental components are missing or altered (Rieseberg and Ellstrand 1993). In a chemosystematic study, it was reported that novel compounds were observed in 46% of the hybrids analyzed (Rieseberg and Ellstrand 1993). The immunologic approach used to assess the concentration of ZR does not allow us to discriminate between ZR and closely related compounds.

An important component in how roots and shoots are integrated is the possibility that chemical signals from

Table 3. *In vitro* leaf disc growth assay. Leaf disks were excised from *Phaseolus* cv. Batt and cv. Redkloud, or F1 hybrid plants and incubated on solutions of (a) KCl plus sucrose (10 mM each), (b) 2× concentrated sap collected from Redkloud or Batt roots, or (c) on zeatin riboside.

Float media	Source of leaf disc	Final disc size (mm)	% Inc size	Significance
KCl plus sucrose	Batt	9.16 ± 0.11	36.8	
	Redkloud	8.53 ± 0.14	27.3	
	F1 hybrid	7.86 ± 0.11	17.3	
Sap from Batt	Batt	9.00 ± 0.08	25.6	NS
	Redkloud	7.99 ± 0.16	19.3	*
	F1 hybrid	7.88 ± 0.13	16.4	NS
Sap from Redkloud	Batt	9.47 ± 0.08	41.3	*
	Redkloud	9.81 ± 0.20	46.4	**
	F1 hybrid	8.55 ± 0.21	27.6	*
ZR (10^{-5}M)	Batt	9.29 ± 0.08	38.7	NS
	Redkloud	8.73 ± 0.15	30.3	NS
	F1 hybrid	8.88 ± 0.13	32.5	**

Data are means for 20–25 discs (±SE). Statistical significance of float media on disc growth was tested by *t* test with * for $p = 0.05$ and ** for $p = 0.001$.

Initial disc diameter was 6.7 mm

roots can influence shoot responses (Bates and Hall 1981, Blackman and Davies 1985, Gollann et al. 1986, Saab and Sharp 1989). A general view has emerged that a change in the concentration of cytokinins transported from the root can elicit shoot responses, although the specific details of this process are not known. On the basis of our studies and those reported in the literature, we hypothesized that altered cytokinin transport from roots to shoots could influence growth and development in crosses between *Phaseolus* of Mesoamerican and Andean origin. In this study, cytokinins were reduced in F1 hybrids. When graft unions were made between roots of Redkloud (Mesoamerican landrace) and F1 hybrids, growth was enhanced and cytokinin concentrations increased in tissues. Our data suggest, therefore, that hybrid weakness associated with F1 hybrids of beans from different centers of origin may be associated with changes in the concentration and/or transport of cytokinins. Although the results presented here indicate ZR flux out of F1 roots is clearly decreased, increased ZR concentrations have been measured in the stems of F1 hybrids (see this issue, “Hybrid Weakness in *P. vulgaris* L. I. Disruption of Developmental and Hormonal Allocation”). Because adventitious roots develop profusely along stems of F1 hybrids, and roots have been suggested as a source of cytokinins (Incoll et al. 1990), it is possible that the ZR found in F1 stems derives not from the primary root system but from the newly developing adventitious roots. This would suggest that roots have the capacity to produce ZR early in development. We are investigating this possibility.

Our data also indicate that xylem sap collected from roots of Redkloud plants can increase the growth of leaf discs excised from F1 hybrids, further supporting the idea that that there may be factors in xylem sap to sustain leaf growth. Others have proposed a role for cytokinins in hybrid weakness. Inoue et al. (1997) found that the culture of F1 seeds in media containing cytokinins could overcome the hybrid lethality resulting between two species of *Nicotiana*.

In addition to measurements of endogenous concentrations of cytokinins in F1 hybrids, we applied cytokinins to roots of F1 plants in a replacement study. The application of BAP mimicked the effect of roots on shoot growth by increasing the number of leaves. Taken together, these results demonstrate that cytokinins may provide a signal for increasing leaf growth. In addition, the results of the leaf disc growth assay provide evidence for the presence of a signal in xylem sap that influences leaf growth. The effects of Redkloud sap on enlargement of discs excised from F1 hybrids suggest that some compound might be present in sap that supports leaf enlargement. The results of these experiments, however, do not allow us to discriminate between actual sap effect and some limitation to leaf enlargement associated with the leaf discs themselves. In addition, we cannot determine the differences between a growth enhancement and an inhibition effect with these data. To clarify the results, we will need to repeat the disc study using discs from some other plant, such as tobacco.

Our study confirms previous reports that crosses between Mesoamerican and Andean genotypes of bean can result in hybrid weakness and further suggests cytokinins may play a role in the hybrid weakness found in this cross. On the basis of the grafting study by Shii et al. (1981) and those reported here, we confirm that the DL system appears to be a novel system in which root-shoot interaction can be studied with a grafting approach and in which cytokinins can be studied. Dissection of the signaling events between roots and shoots is essential in understanding the mode of action of this poorly understood class of plant hormones.

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