2005

Cytokinin Accumulation and an Altered Ethylene Response Mediate the Pleiotropic Phenotype of the Pea Nodulation Mutant R50 (*sym16*)

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Abstract: R50 (*sym16*), a pleiotropic mutant of *Pisum sativum* L., is short, has thickened internodes and roots, and has a reduced number of lateral roots and nodules. Its low nodule phenotype can be restored with the application of ethylene inhibitors; furthermore, it can be mimicked by applying cytokinins (CKs) to the roots of the parent line 'Sparkle'. Here, we report on the etiolation phenotypes of R50 and 'Sparkle', and on the interactive roles of ethylene and CKs in these lines. R50 displayed an altered etiolation phenotype, as it was shorter and thicker, and had more developed leaves than dark-grown 'Sparkle'. Shoot morphological differences induced by exogenous ethylene or CKs were found to be less severe for R50. Ethylene inhibitor application induced root and shoot elongation and encouraged apical hook opening in both etiolated lines. Liquid chromatography – tandem mass spectrometry analysis indicated that CK concentrations in R50 were higher than in 'Sparkle', particularly in mature shoots where the levels were maintained at elevated concentrations. These differences indicate a reduction in the CK catabolism of R50. The accumulation of CKs can be directly related to several traits of R50, with the reduced number of nodules and altered shoot ethylene response being likely indirect effects.

Key words: cytokinin, de-etiolation, ethylene, etiolation, pea, nodulation mutant.

Résumé : Le R50 (*sym 16*), un mutant pléïotrope du *Pisum sativum* L., est court, possède des entre-nœuds et des racines épaissies ainsi qu'un nombre réduit de racines latérales et de nodules. Son phénotype à faible nodulation peut être restauré par l'application d'inhibiteurs de l'éthylène; on peut de plus le reproduire par l'application de cytokinines (CKs) aux racines de la lignée parentale 'Sparkle'. Les auteurs font ici état des phénotypes d'étiolement du R50 et de la lignée parentale 'Sparkle', sur les rôles interactifs de l'éthylène et des CKs sur ces lignées. Le R50 montre une altération du phénotype d'étiolement, étant plus court, plus épais, et développant plus de feuilles que le 'Sparkle' cultivé à l'obscurité. On constate que les différences morphologiques de la tige, induites par l'éthylène exogène ou les CKs, sont moins sévères chez le R50. Une application d'inhibiteurs de l'éthylène induit l'élongation des tiges et des racines et encourage le déroulement de la courbe apicale chez les deux lignées étiolées. Les analyses de chromatographie en phase liquide – spectrométrie de masse en tandem indiquent que les teneurs en CKs sont plus élevées chez le R50 que chez le 'Sparkle', surtout chez les tiges matures où les teneurs se maintiennent élevées. Ces différences indiquent une réduction du catabolisme des CKs chez le R50. L'accumulation de CKs peut être directement reliée à plusieurs caractères du R50, la réduction du nombre de nodules et la réaction modifiée de la tige à l'éthylène étant vraisemblablement des effets indirects.

Mots clés : cytokinine, désétiolement, éthylène, étiolement, pois, mutant de la nodulation.

[Traduit par la Rédaction]

Introduction

Mutants have proven to be useful tools for determining the roles of various signalling components in plants. R50 (*sym16*), a pleiotropic mutant of *Pisum sativum* L. 'Sparkle' (pea), was originally selected for its inability to nodulate properly (Kneen and LaRue 1988). It has a short stature, thick internodes and roots, fewer and shorter lateral roots, and a lower chlorophyll content in young leaves (Guinel and Sloetjes 2000). While most of these characteristics suggest a role for ethylene in the R50 phenotype, others traits such as wrinkled leaves (Kneen et al. 1994) and abnormal develop-

Received 20 October 2004. Published on the NRC Research Press Web site at http://canjbot.nrc.ca on 21 September 2005.

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 doi: 10.1139/B05-049

ment of vascular tissue (Ferguson 2002) indicate a potential role for cytokinins (CKs; e.g., Rupp et al. 1999; Werner et al. 2003).

In R50, nodule development is blocked early; both infection threads and nodule primordia fail to progress properly (Guinel and Sloetjes 2000). Interestingly, treating the parent line 'Sparkle' with exogenous CKs induces a block in nodule organogenesis comparable to that of R50 (Lorteau et al. 2001), suggesting a role for CKs in the R50 nodulation phenotype. If R50 has an overabundance of CKs, an overproduction of ethylene would be expected (e.g., Lorteau et al. 2001). Indeed, R50 appears either to overproduce or to be overly sensitive to ethylene as root treatment with ethylene inhibitors restores nodule numbers to those of 'Sparkle' (Guinel and Sloetjes 2000). To date, the involvement of CK in nodulation has been investigated mainly by the application of exogenous compounds (e.g., Lorteau et al. 2001), and more recently by the use of a transgenic model plant (*Lotus japonicus*) that overproduces the degradative enzyme cytokinin oxidase (CKX; Lohar et al. 2004). Thus, R50 could not only aid in the identification of the role of ethylene in nodulation, but it could also be an effective tool for studying the role(s) of CKs in this symbiotic process.

In this paper, we demonstrate that R50 accumulates CKs and uncouple the roles of CKs and ethylene in the R50 phenotype by growing the plants in the dark. R50 displayed a reduced etiolation phenotype. Its shoots were less sensitive to exogenous ethylene than those of 'Sparkle', whereas the roots of both pea lines were similar in their ethylene response. These findings may be explained by the endogenous concentrations of CKs, which were elevated in R50 seedling roots and shoots and maintained at high levels in the shoot as the plant matures.

Materials and methods

Plant growth conditions

'Sparkle' and R50 seeds were surface-sterilized in an 8% (*v*/*v*) bleach solution (5.25% NaOCl) for 4 min followed by three 3-min rinses in sterilized water; they were placed in water and left overnight in darkness to imbibe. Unless stated otherwise, they were then planted individually in Conetainers™ (66 mL, 2.5 cm × 16 cm, Stuewe & Sons Inc., Corvallis, Oregon) filled with sterile vermiculite (Holiday, Vil Vermiculite Inc., Toronto, Ontario). The Conetainers™ were wrapped in aluminium foil to exclude light from the roots and held in 1-L beakers (dark treatment) or trays (light treatment) containing water. Plants were grown either under continuous darkness (temperature regime explained later) for the etiolation studies or under a 23 °C : 18 °C, 16 h : 8 h light:dark regime in a growth-room under incandescent (OGE 600 h, 60–120 W, 120 V, General Electric) and cool white fluorescent lights (Watt-Miser GE, F96TIZ-CW-HO-WM), yielding a photosynthetic photon flux density of 280 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Etiolation characterization

To determine the effect of ethylene on R50, 10 mL of either water (control) or the immediate ethylene precursor ACC (1-amino-cyclopropane 1-carboxylic acid; Sigma-Aldrich Canada Ltd, Oakville, Ontario; 1 $mmol \cdot L^{-1}$ made up in water) were administered. Treatments were applied 3, 6, and 9 d after planting (DAP) to the vermiculite surface of plants held in either 16 h : 8 h light:dark or continuous darkness. Treatments of the plants growing in the dark were made under green light (40 W, 120 V, Decocolour Solid, General Electric). Light-grown plants were subjected to a temperature regime of 23 °C : 18 °C, whereas those in darkness were grown at a temperature of 21 °C. After 5, 8, or 11 DAP, shoot and root lengths, number of lateral roots, and epicotyl hook angle were measured. The hook angle was measured as the angle between the vertical base of the epicotyl and the apical portion. The shoots and roots were then excised, dried at 60 °C for 3 d, and weighed. For each parameter, at least 6 control- and 6 ACC-treated plants were assessed in each of three repeated experiments.

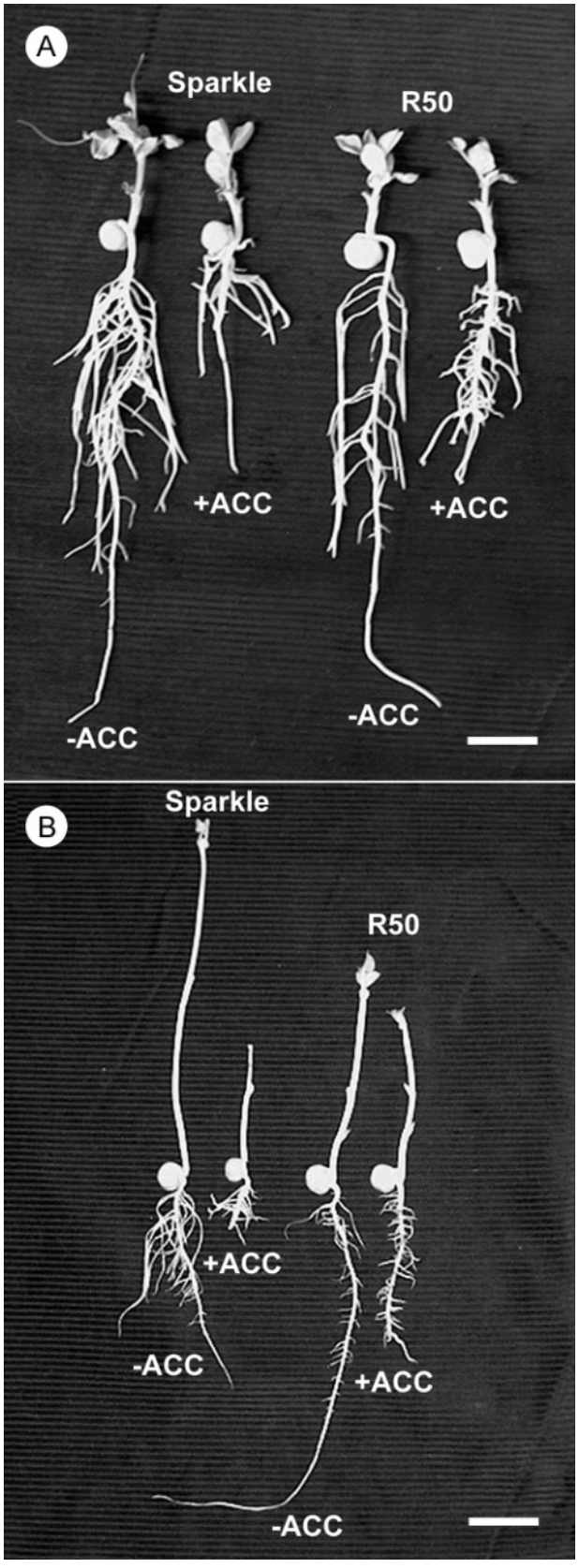
The effects of CKs and ethylene inhibitors on etiolated plants were determined by treating dark-grown seedlings at 3, 6, and 9 DAP with 10 mL of either water or water with a drop of 100% ethanol (control), the synthetic CK benzyl aminopurine (BAP, 10 $\mu mol \cdot L^{-1}$ initially dissolved in a drop of 100% ethanol; Sigma-Aldrich), or the ethylene inhibitor aminoethoxy vinyl glycine (AVG, 10 $\mu mol \cdot L^{-1}$; Sigma-Aldrich). The seedlings were harvested at 5, 8, and 11 DAP, respectively, and the same parameters as above were measured. Two separate experiments using 12 control and 12 treated plants were performed, but at different times of the year, in a darkroom not equipped with a temperature control. As a consequence, the plants grown in the winter were shorter than the plants grown in the summer. Because of this difference in height, data were standardized for comparative purposes (see statistical analysis).

Exogenous ethylene treatment

Imbibed seeds were set on wet sterile Petri plates for 2 d before being planted in vermiculite-filled Conetainers™ (560 mL). These were placed into a wood frame that was fitted and sealed atop an opaque-plastic chamber (125 L) in such a way that the roots of the seedlings could be treated with ethylene, while their shoots remained in open air. Exogenous ethylene was applied continuously to 'Sparkle' and R50 according to the methods of Lee and LaRue (1992) as modified by Geil et al. (2001). Four chambers received a continuous flow of either 0, 1.1, 2.5, or 5.7 µL/L ethylene. The ethylene gas, adjusted by microvalves (Nupro, Willoughby, Ohio) and mixed with air, was released into the water that filled the bottom of the chamber. The flow-rate through each line was adjusted to 2 L/min using a flow meter (7631T-603; Matheson Gas Products, Whitby, Ontario).

Throughout the experiment, the concentration of ethylene around the roots in each chamber was monitored. One-millilitre air samples were collected from 14-cm-long, 0.635-cm internal diameter, septum-capped PVC tubes that had been inserted into one Conetainer™ during the setup of each chamber. The samples were injected manually into a Perkin-Elmer 3920B gas chromatograph (GC; Perkin-Elmer, Norwalk, Connecticut) equipped with a flame ionization detector. The GC was fitted with a 1.83-m stainless steel column (0.3175-cm outer diameter) packed with Porapak N (80/100 mesh; Chromatographic Specialists Inc., Brockville, Ontario). The column temperature was held at 115 °C and the carrier gas (He) flow rate was held at 23 mL/min; the

Fig. 1. Eleven-day-old *Pisum sativum* 'Sparkle' and R50 (*sym16*) treated with either water (–ACC) or 1 mmol·L^{-1} ACC (+ACC) under either a 16 h : 8 h light:dark regime (A; scale bar = 1.74 cm) or continuous darkness (B; scale bar = 2.20 cm).



head pressure was 42 psi (1 psi = 6.895 kPa). Samples of air from the growth room were also analysed to ensure that the air surrounding the shoots was free of ethylene. The morphological parameters mentioned above, and the diameter of the third internode, were measured at 5, 8, and 11 DAP. At least 10 plants were sampled in each of three different experiments.

Cytokinin extraction and separation

CKs were extracted from plants harvested at 9 and 17 DAP, and separated under conditions established by Emery et al. (1998) to prevent or minimize enzyme activity causing CK nucleotide degradation and CK isomerization. Frozen root or shoot (6–10 g fresh mass (FM)) samples were homogenized (Ultra-Turrax T8; IKA-Werke GmbH, Staufen, Germany) over ice in cold (–20 °C) modified Bieleski extraction buffer (15:4:1 CH_3OH:H_20:HCOOH, by volume) at 5 mL·g^{-1} FM. One hundred nanograms of each of the following deuterated CKs, [$^2H_6$]iP, [$^2H_6$][9R]iP, *trans*-[$^2H_5$]Z, [$^2H_3$]DZ, *trans*-[$^2H_5$][9R]Z, [$^2H_3$][9R]DZ, [$^2H_6$][9R-MP]iP, and [$^2H_6$][9R-MP]DZ (Apex Organics, Honiton, Devon, UK), were added as internal standards for quantification of endogenous CKs using the isotope dilution method. Samples were allowed to extract passively overnight at –20 °C, and solids were recovered by centrifugation (24 000*g*, 15 min, 4 °C) and re-extracted for 30 min in extraction buffer at –20 °C. Pooled supernatants were dried in vacuo at 40 °C and residues were reconstituted in 5 mL of 1.0 mol·L^{-1} formic acid. Extract pH was verified to be <2.8 to ensure CKs were entirely cations. Extracts were purified according to Dobrev and Kaminek (2002) on a mixed mode reverse-phase-cation-exchange Oasis MCX-SPE column (Waters, Mississauga, Ontario), preconditioned with 5 mL of CH_3OH, followed by 5 mL of 1.0 mol·L^{-1} HCOOH. Once the sample was loaded, the column was washed with 5 mL of 1.0 mol·L^{-1} HCOOH, followed by 5 mL of CH_3OH. CK nucleotides were first eluted with 5 mL of 0.35 mol·L^{-1} NH_4OH; and then the CK free bases and ribosides were eluted with 5 mL of 0.35 mol·L^{-1} NH_4OH in 60% (*v*/*v*) CH_3OH. All samples were dried in vacuo at 40 °C. Three independent samples from three different harvests were analysed for every datum.

For quantification, nucleotides eluted from the MCX-SPE column were degraded to nucleosides by incubation with 3.4 units of alkaline phosphatase (P 4252; Sigma-Aldrich) in 1 mL of 0.1 mol·L^{-1} methanolamine-HCl (pH 10.4) for 12 h at 37 °C (Emery et al. 2000). Resultant nucleosides were dried in vacuo and reconstituted in double-distilled water for further purification on a reversed-phase C_{18} column (500 mg, AccuBOND ODS; Fisher Scientific, Mississauga, Ontario) preconditioned with 20 mL of double-distilled water, followed by 10 mL of CH_3OH. After loading the sample, the column was washed with 20 mL of double-distilled water. Nucleosides were eluted with 20 mL of 80:20 (*v*/*v*) methanol : double-distilled water and dried in vacuo at 40 °C.

LC (+ES) MS/MS quantification of endogenous cytokinins

CKs from tissue samples were separated and analyzed by a Waters 2680 Alliance HPLC system (LC; Waters, Milford, Massachusetts) linked to a Quattro-LC triple quadrupole mass spectrometer (MS; Micromass, Altrincham, UK) equipped with

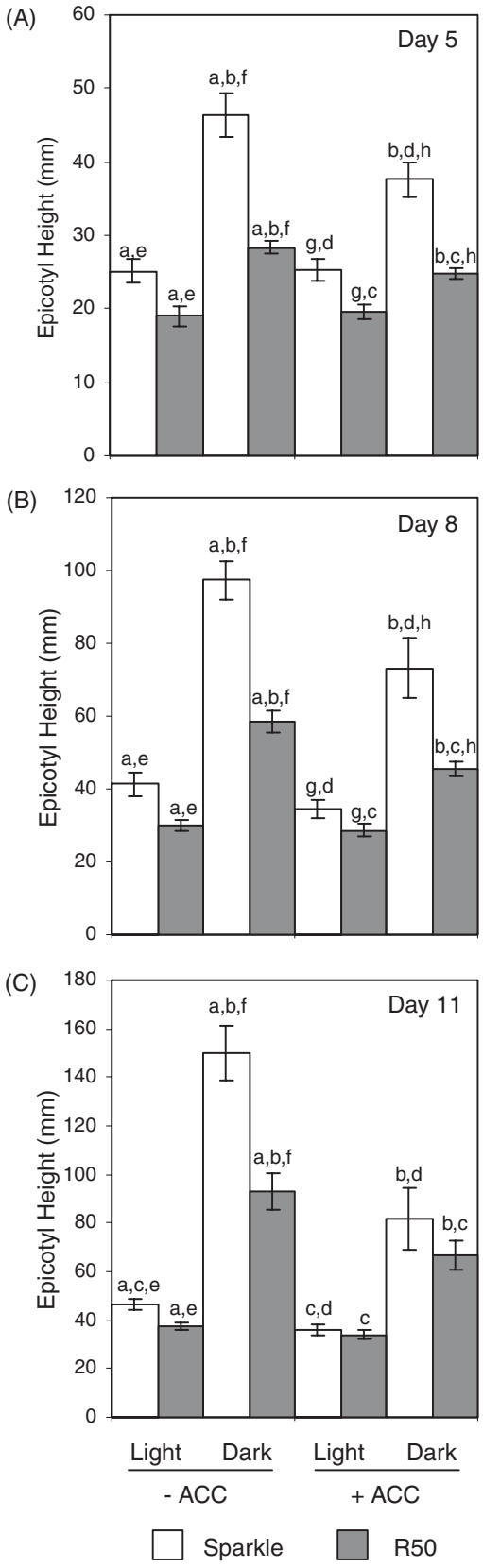


Fig. 2. Epicotyl height of two *Pisum sativum* lines at different ages (A, day 5; B, day 8; C, day 11) and under different growth conditions (treated without (only water; –ACC) or with ACC (1 mmol·L^{-1}; +ACC) at 3, 6, and 9 d after planting in either light or dark conditions). Values are means ± SE from all replicates (n = 18). Means with the same letters indicate significant treatment effects within a pea line (a–d) or significant effects of pea line within a treatment (e–h).

a Z-spray ionization source. Positive electrospray (+ES) interface was used for all analyses (LC (+ES) MS/MS). A sample volume of 20 µL was injected into a Genesis C_{18} reversed-phase column (4 µm, 150 mm × 2.1 mm; Jones Chromatography, Foster City, California). CKs were eluted with an increasing gradient of acetonitrile (A) mixed with 0.1% formic acid in 20 mmol·L^{-1} ammonium acetate (*v*/*v*; pH adjusted to 4.0) (B) at a flow rate of 0.2 mL/min. The initial conditions were 8% A and 92% B, changing linearly after 5 min to 15% A and 85% B for 2 min, followed by 100% B for 2 min, and then linearly returning back to initial conditions for 2 min. The effluent was introduced into the electrospray source (source block temperature of 80 °C, desolvation temperature of 250 °C), and CKs were quantified by multiple reaction monitoring of the mother (parent) ion and the appropriate daughter (product) ion as in Prinsen et al. (1995).

Statistical analysis

For all morphological experiments where statistics were employed, controls refer to water-treated plants, grown either in light and dark regime or continuously in the dark. For the ACC and dark treatments, one-way analysis of variance (ANOVA) was used to determine any significant difference existing between pea lines and, within each pea line, between treatments. These tests followed a normality test, the outcome of which determined, if necessary, the pairwise multiple comparison procedure used after the ANOVA test. If the normality test passed, the Tukey test was performed; if it failed, the Dunn's method test was used. For the BAP and AVG treatments, two-way ANOVA was used to determine significance between pea lines and, within each pea line, between treatments; these tests were also performed to determine any significant interaction between pea lines and treatments. They were followed, if necessary, by pairwise multiple comparison procedures (Tukey). Only differences with $P \leq 0.05$ were considered significant. Because the two replicate experiments performed to study the effects of BAP and AVG on etiolated seedlings were run at different times of the year, the morphology of the plants was different (see Results). The results, which followed the same trend between experiments, varied enough to require that, for meaningful comparison, each trait was expressed as a percentage of the control. All statistics were performed using SigmaStat® version 2.03 software (SPSS Inc., Chicago, Illinois).

Results

Etiolation characteristics

Control

'Sparkle' responded to continuous darkness as expected (–ACC; Figs. 1–4). The epicotyl was spindly and tall, with the height difference between light- and dark-grown plants increasing as the seedlings became older (Fig. 2). This conspicuous increase in size was not accompanied by a similar increase in dry mass (DM); by day 11, the DM of the etio-

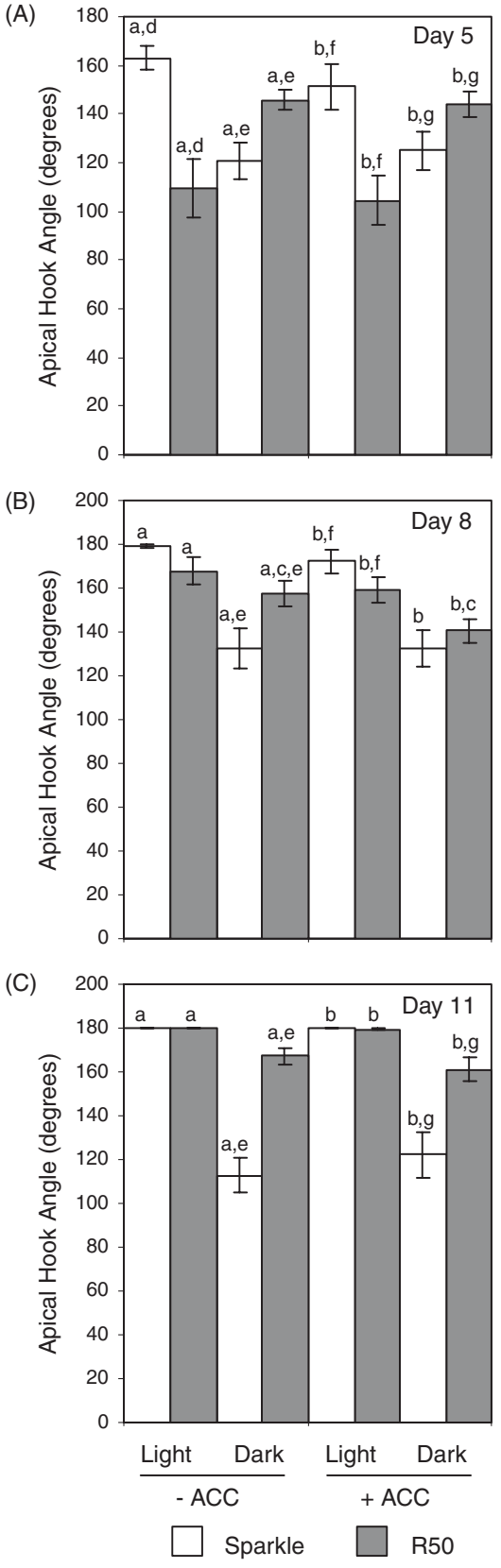


Fig. 3. Apical hook angle of seedlings of two *Pisum sativum* lines at different ages (A, day 5; B, day 8; C, day 11) and under different growth conditions (treated without (only water; –ACC) or with ACC (1 mmol·L^{-1}; +ACC) at 3, 6, and 9 d after planting in either light or dark conditions). Values are means ± SE from all replicates (n = 18). Means with the same letters indicate significant treatment effects within a pea line (a–c) or significant effects of pea line within a treatment (d–g).

lated seedlings was in fact significantly smaller than those of light-grown seedlings (39 ± 4 and 50 ± 6 mg, respectively). In addition, light-grown seedlings exhibited fully opened apical hooks at 8 DAP, whereas dark-grown seedlings had relatively closed hooks even at 11 DAP (Fig. 3). By 11 DAP, the leaves of etiolated plants were chlorotic and poorly developed whereas those of light-grown seedlings were green and fully formed, but not yet mature (Fig. 1). No differences were seen in the primary root length of light- and dark-grown 'Sparkle' seedlings (Fig. 4). However, the DM of the entire root system of etiolated plants was less than that of light-grown plants (26 ± 1 and 45 ± 4 mg, respectively, at 11 DAP); these differences increased as the plants aged. Lateral root numbers did not account for these DM variations as the number of roots increased in the dark (40 ± 3 versus 27 ± 3 in the light at 11 DAP).

For all parameters measured, except apical hook curvature, the differences between light- and dark-grown R50 (Fig. 1) were similar to those of 'Sparkle', but smaller in scale (Figs. 2 and 4). Etiolated R50 was taller than light-grown R50, but shorter and thicker than dark-grown 'Sparkle' (Figs. 1 and 2). In light-grown conditions, there was no difference between the primary root lengths of R50 and 'Sparkle' (Figs. 1 and 4); however, the DM of the R50 root system was smaller (38 ± 2 and 45 ± 4 mg for R50 and 'Sparkle', respectively, at 11 DAP). Dark conditions did not alter these two parameters in R50 (Fig. 4), although the lateral root number doubled (20 ± 1 to 44 ± 3 at 11 DAP). In contrast, the apical hooks of R50 seedlings grown in the dark were much more open than those of 'Sparkle', whereas in the light, they were much less open (Fig. 3). Furthermore, although chlorotic, the leaves of dark-grown R50 were much more developed than those of dark-grown 'Sparkle' (Fig. 1).

Ethylene precursor treatment

'Sparkle' and R50 seedlings responded differently to the ACC treatment, which effectively resulted in the production of endogenous ethylene. In the light, ACC-treated 'Sparkle' exhibited a shorter epicotyl (Fig. 2), a slightly more exaggerated apical hook (Fig. 3), and a smaller root system with a shorter primary root than control (i.e., light-grown, non-ACC-treated) seedlings (Figs. 1A and 4). The number of lateral roots was not altered by the ACC treatment (about 30 by 11 DAP). In the dark, ACC-treated 'Sparkle' was also much shorter than nontreated plants (Figs. 1B and 2), and this difference in epicotyl height increased as the seedlings grew older (Fig. 2). The epicotyl DM of etiolated ACC-treated 'Sparkle' was also smaller than that of ACC-treated seedlings grown in the light (data not shown). The apical hook of dark-grown, ACC-treated 'Sparkle' was as accentuated as that of etiolated, nontreated 'Sparkle', but more exaggerated than that of seedlings grown in the light, with or without ACC (Fig. 3). The primary root of dark-grown, ACC-treated 'Sparkle' decreased in length (Fig. 4), but not in the number of emerged secondary roots it bore (i.e., 32 ± 3 by 11 DAP). In addition, the difference observed in the root system DM

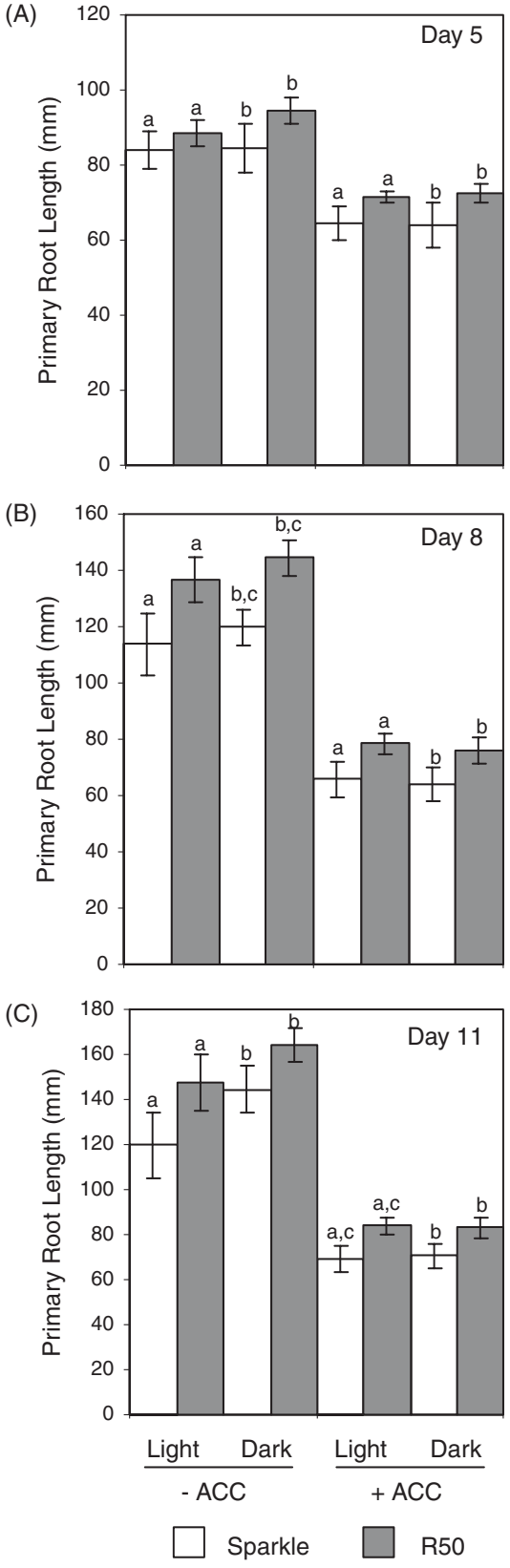


Fig. 4. Primary root length of seedlings of two *Pisum sativum* lines at different ages (A, day 5; B, day 8; C, day 11) and under different growth conditions (treated without (only water; –ACC) or with ACC (1 mmol·L^{-1}; +ACC) at 3, 6, and 9 d after planting in either light or dark conditions). Values are means ± SE from all replicates (n = 18). Means with the same letters indicate significant treatment effects within a pea line (a and b) or significant effects of pea line within a treatment (c).

between the control and the treatment increased with age (data not shown).

Although R50 responded to the presence of ACC, its response was not as strong as that of 'Sparkle' (Figs. 1–4). In the light, there were no differences in epicotyl height between non-ACC-treated and ACC-treated seedlings (Figs. 1 and 2). In contrast, there was a decrease in primary root length (Fig. 4) that was accompanied by an increase in lateral root number (from 20 ± 1 without ACC to 37 ± 3 with ACC at 11 DAP), resulting in similar root system DM. Furthermore, as in 'Sparkle', ACC did not affect the opening of the apical hook, which by 11 DAP had completely opened in all plants (Fig. 3). In dark conditions, as in the light, R50 was not as strongly affected by ACC as 'Sparkle' (Figs. 2–4). ACC-treated R50 epicotyls were reduced in height and DM, but these reductions were less pronounced than those for treated 'Sparkle' (Figs. 1B and 2). Furthermore, the curvature of the apical hook of ACC-treated R50 was more exaggerated than that of nontreated light-grown R50 (Fig. 3), but less than that of either nontreated or treated etiolated 'Sparkle' at all ages examined (Fig. 3). As in the light, ACC treatment decreased the length of R50 primary roots by half (Fig. 4), but had no effect on the number of lateral roots or root system DM (data not shown).

CK treatment

CKs are known to promote the synthesis of endogenous ethylene in pea seedlings grown either in a light and dark regime (Lorteau et al. 2001) or in etiolating conditions (Bertell and Eliasson 1992). BAP treatment was thus expected to give results similar to those obtained with ACC treatment.

By 11 DAP, the epicotyl of BAP-treated, dark-grown 'Sparkle' was much shorter than that of the control, but the curvature of the apical hooks was similar (Table 1). BAP affected neither the emergence nor the growth of lateral roots. The entire root system had a larger mass than that of nontreated etiolated 'Sparkle' (data not shown) even though 'Sparkle' primary roots were shortened (Table 1).

Dark-grown R50 responded differently to BAP than did 'Sparkle'. At 11 DAP, the epicotyl of R50 was almost unresponsive but its primary root was shorter (Table 1), although the number of lateral roots and the root DM were not altered (data not shown). Furthermore, the curve of the apical hook of R50 was less exaggerated than even that of nontreated 'Sparkle' seedlings (Table 1).

Ethylene inhibitor treatment

Both lines responded to AVG in a similar manner (Table 2); however, the effect of AVG was less evident in R50 than in 'Sparkle'. Epicotyl height and primary root length increased (Table 2), as did their DMs (data not shown). The number of lateral roots was unaltered, and the apical hook curvature was less exaggerated than in nontreated seedlings (Table 2).

Table 1. Effect of 10 μmol·L^{-1} BAP treatments on two *Pisum sativum* lines ('Sparkle' and R50) grown under dark conditions.

		'Sparkle'		R50	
	Control mean[a]	0 BAP	10 μmol·L^{-1} BAP	0 BAP	10 μmol·L^{-1} BAP
Epicotyl height[†]	10.34±0.83 cm	1.00±0.07	0.69±0.06*	0.68±0.05	0.60±0.05
Apical hook angle	131.53±12.34 degrees	1.00±0.09	0.97±0.06	1.14±0.08	1.12±0.06
Primary root length	15.78±1.52 cm	1.00±0.10	0.77±0.06*	1.23±0.07	0.72±0.05*

Note: Parameters were measured on 11-d-old seedlings that received treatments at 3, 6, and 9 DAP. Two-way ANOVA was performed and significant treatment and line effects are denoted by * and †, respectively.

[a]Means ± SE of control dark-grown, nontreated 'Sparkle' from both summer and winter replicates (n = 17). All other values are expressed as a percentage (100% = 1) of this value.

Table 2. Effect of 10 μmol·L^{-1} AVG treatments on two *Pisum sativum* lines ('Sparkle' and R50) grown under dark conditions.

		'Sparkle'		R50	
	Control mean[a]	0 AVG	10 μmol·L^{-1} AVG	0 AVG	10 μmol·L^{-1} AVG
Epicotyl height[†]	8.02±1.03 cm	1.00±0.14	1.23±0.13	0.71±0.07	0.85±0.11
Apical hook angle[†]	113.06±9.27 degrees	1.00±0.07	1.27±0.06	1.19±0.07	1.45±0.09*
Primary root length	14.19±1.45 cm	1.00±0.10	1.01±0.06	1.03±0.04	1.10±0.07

Note: Parameters were measured on 11-d-old seedlings that received treatments at 3, 6, and 9 DAP. Two-way ANOVA was performed and significant treatment and line effects are denoted by * and †, respectively.

[a]Means ± SE of control dark-grown, nontreated 'Sparkle' from both summer and winter replicates (n = 17). All other values are expressed as percentages (100% = 1) of these values.

Exogenous ethylene treatment

Exogenous ethylene treatment did not improve the similarity between the two lines. When the rhizosphere was continuously treated with a constant, relatively low amount of ethylene (1.1 μL/L), 'Sparkle' seedlings were reduced in size (Figs. 5A and 6A). By 11 DAP, the epicotyl was about 30% shorter than that of nontreated plants (Fig. 5A), the diameter of the third internode was almost 45% bigger (Fig. 5B), and the curvature of the apical hook was more exaggerated (Fig. 5C). Primary root length (Fig. 6A), lateral root number (Fig. 6B), and lateral root elongation (Fig. 6C) were all reduced (35%, 55%, and 20%, respectively). The severity of the effects on 'Sparkle' increased as the concentration of applied ethylene was increased to 5.7 μL/L (Figs. 5 and 6).

As was observed in R50 etiolated seedlings treated with ACC and BAP, the epicotyl of R50 was much less responsive than that of 'Sparkle', whereas its root system was as responsive. However, the response of R50 to exogenous ethylene was quantitatively different from that of 'Sparkle' (Figs. 5 and 6). Although there was a decrease in the epicotyl height of treated versus nontreated R50 seedlings, the differences induced by the lowest and the highest ethylene concentrations were less than those observed in 'Sparkle' (Fig. 5A). A similar observation was made for the curvature of the apical hook of treated plants, which was much less exaggerated than that of the treated 'Sparkle' seedlings (Fig. 5C). Furthermore, the third internode of R50 increased in diameter upon ethylene treatment (Fig. 5B); however, the difference between treated and nontreated seedlings was much less pronounced for R50 than it was for 'Sparkle'. The root system of R50 was responsive to ethylene; for instance, the length of R50 primary roots was similar to that of 'Sparkle' when treated with the higher concentrations of ethylene (Fig. 6A). By 11 DAP, the length of R50 primary roots was reduced by more than 60%, and the number of lateral roots by 40%, but the length of the longest lateral roots was not different from that of the control (Fig. 6C).

Cytokinin identification and quantification

The concentrations of various CKs present in 'Sparkle' and R50 plants at 9 and 17 DAP are shown in Tables 3 and 4, respectively. At 9 DAP (Table 3), the most abundant CK group in shoots and roots of both lines was the nucleotides: *trans*-[9R-MP]Z, *cis*-[9R-MP]Z, [9R-MP]DZ, and iP-NT. The most abundant riboside was [9R]iP, with *trans*-[9R]Z, *cis*-[9R]Z, and [9R]DZ present in smaller quantities. By 17 DAP (Table 4), the riboside-CK abundance was more similar to that of the nucleotides. At both times, the free base forms, such as *cis*- and *trans*-Z and DZ, were either in very low abundance or not detected. Relatively low concentrations of iP were seen, but this reflected its superior recovery rates compared with those of the other free bases. This progression of lowered abundance from nucleotide to riboside and then to free base is consistent with the pathways of CK biosynthesis recently proposed by Sakakibara and Takei (2002), whereby any of the nucleotides, which are early pathway forms, can be converted to their ribose equivalents in a parallel ribosyl-CK pathway. The free bases, following the further conversion of the ribosides, are regarded as late pathway members.

At 9 DAP, CK profiles between 'Sparkle' and R50 were qualitatively quite similar (Table 3), and total CK concentrations were comparable (Fig. 7), although some notable quantitative differences in specific forms of CK were apparent. For example, the concentration of the nucleotide [9R-MP]DZ in R50 roots was twice that in 'Sparkle' (Table 3). This difference was further reflected in [9R]DZ, which was abundant in R50 but detected only at trace concentrations in 'Sparkle' (Table 3). In the shoots, iP-NT and [9R]iP concentrations were both greater in R50 than in 'Sparkle' (Table 3), but there were no major differences between the two lines in

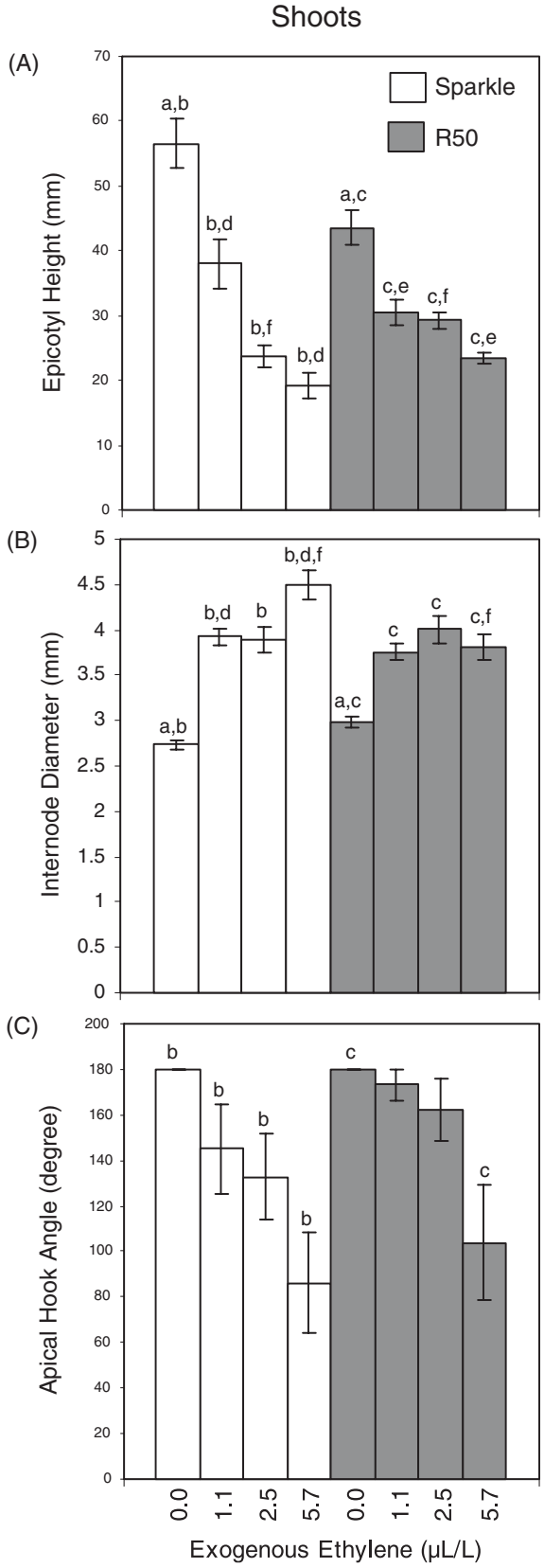


Fig. 5. Epicotyl height (A), third internode diameter (B), and apical hook curvature (C) of *Pisum sativum* 'Sparkle' and R50 seedlings, the roots of which were continuously treated with exogenous ethylene. Measurements were made on 11-d-old seedlings; values are means ± SE (n = 32). Statistical comparisons between the two untreated pea lines (significant differences annotated a), the control and treatments of each line (significant differences annotated b and c, for 'Sparkle' and R50, respectively), treatments within a line (significant differences annotated d and e, for 'Sparkle' and R50, respectively), and the two pea lines treated with the same concentration of ethylene (significant differences annotated f) are indicated.

unsaturated side-chain hydroxylated CKs (i.e., those presumed active).

By 17 DAP, the total CK concentration generally decreased in roots of both lines (Fig. 7), especially the CK-nucleotides and [9R]iP (compare Table 3 to Table 4). However, the concentration of total CK nucleotides was markedly higher in R50. This was specifically reflected in the concentrations of *cis*- and *trans*-[9R-MP]Z, and [9R-MP]DZ, which were all greater in R50 than in 'Sparkle'. Greater differences between R50 and 'Sparkle' were observed in the shoots where the total CK in R50 remained at a similar level from 9 to 17 DAP (compare Table 3 to Table 4), whereas that of 'Sparkle' was reduced by more than 50% (Fig. 7). Thus, by 17 DAP the total CK level of R50 shoots was approximately three times that of 'Sparkle', and the total plant CK level was more than double (Fig. 7).

Discussion

Although R50 was originally selected as a nodulation mutant, its phenotype is quite complex, suggesting that the altered gene product, SYM16, affects many aspects of vegetative development, including nodule organogenesis. The overall higher concentrations of CKs that were detected in the mutant, particularly in its mature shoot, make R50 extremely valuable because it is the first legume mutant to display this trait. We propose a direct link between high endogenous CK concentration and the partial etiolation phenotype of R50, and an indirect link to its altered ethylene response.

The etiolation phenotype and CK profile of R50

Because dark-grown seedlings of R50 have a variety of characteristics normally associated with light-grown plants, R50 exhibits an etiolation phenotype, albeit partial. Another pea mutant, *lip1*, has previously been characterized as displaying a de-etiolation phenotype (Frances et al. 1992). Light-grown *lip1* plants are shorter and darker green than the wild type; although their fertility is reduced, they flower and senesce at the same time as the wild type (Frances et al. 1992). R50 is different from *lip1* when grown in the light as its epicotyls are pale and it has normal fertility despite its delayed timing of flowering and senescence. Because the *lip1* mutant has been shown to be deficient in a photomorphogenesis repressor (Frances and Thompson 1997), it is probably more relevant to compare R50 to the etiolation mutants of *Arabidopsis* that also accumulate CKs.

The *Arabidopsis* de-etiolation mutant *det1* has reduced fertility, a saturated response to CKs and relatively normal concentrations of CKs (Chory et al. 1994). Other *Arabidopsis* de-etiolation mutants include *amp1*, which has

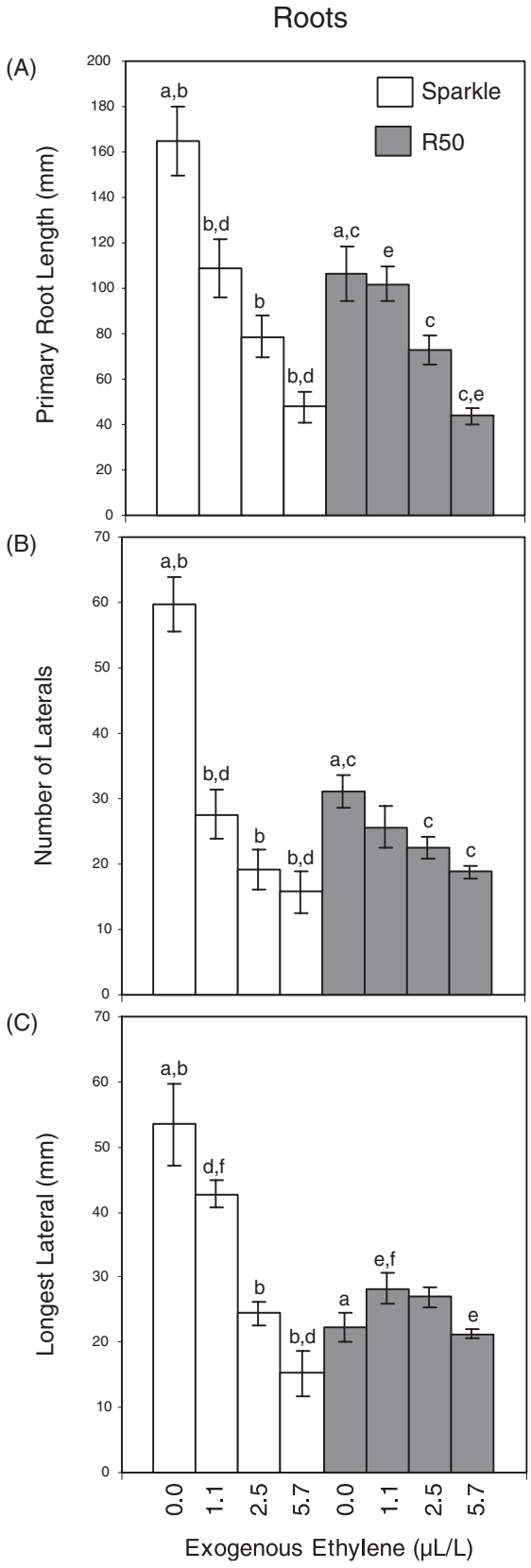


Fig. 6. Primary root length (A), number of lateral roots (B), and length of the longest lateral roots (C) of *Pisum sativum* 'Sparkle' and R50 seedlings, the roots of which were continuously treated with exogenous ethylene. Measurements were made on 11-d-old seedlings; values are means ± SE (n = 32). Statistical comparisons are described in Fig. 5.

slightly elevated concentrations of CKs (Chaudhury et al. 1993; Nogué et al. 2000) and *hoc1*, which displays short epicotyls, a bushy architecture, and a higher number of rosettes in the light (Catterou et al. 2002). *hoc1* is a dark-green mutant that accumulates two and seven times more total CKs in its shoots and roots, respectively, than the wild-type (Catterou et al. 2002). By comparison, the total concentrations of CKs in the shoot and root of R50 were three- and two-fold, respectively, those in 'Sparkle'. An increased CK concentration in R50 could influence the light-regulated signalling pathway, thus contributing to the partial etiolation phenotype of the plant.

We observed greater concentrations of some key CK forms in R50 (Tables 3 and 4, Fig. 7). Firstly, iP-NT concentrations were higher than those of 'Sparkle'. This may be an indication of a greater CK biosynthesis in R50 since iP-NT encompasses the three possible precursor forms in CK biosynthesis (Sakakibara and Takei 2002). In fact, our methods did not distinguish [9R-MP]iP from the two other precursors, [9R-DP]iP and [9R-TP]iP; because of the requirements of LC-MS/MS, all nucleotides must be dephosphorylated to ribosides before analysis. Thus in the nucleotide fraction, [9R-MP]iP, [9R-DP]iP, and [9R-TP]iP were all analyzed as iP-NT. Secondly, [9R]iP was also slightly higher in R50. Thirdly, the CKs with saturated isopentenyl side-chains, [9R]DZ and [9R-MP]DZ, were consistently higher in R50 roots. Such a high level of potentially active dihydro-CK forms would be difficult for the plant to eliminate because these are not substrates of CKX, the enzyme known to degrade CKs (Jones and Schreiber 1997). The result is also novel in that these dihydro-CKs have rarely been detected in root tissues of any species (Emery and Atkins 2002).

The CK profile of R50 shoots at 17 DAP was different from that observed at 9 DAP: the concentrations of nucleotides declined, whereas those of the ribosides remained more or less constant (Tables 3 and 4). In contrast, all CK concentrations decreased in 'Sparkle' shoots. A decline in total CKs was observed in all situations, except in R50 shoots. These findings suggest a slower rate of CK metabolic degradation and (or) elevated CK synthesis in R50. Nucleotide-CKs have long been considered resistant to CKX (Laloue and Fox 1989); however, recently, Werner et al. (2003) observed a significant reduction in [9R-MP]Z and iP-NT in four lines of CKX-overproducing transgenic *Arabidopsis*. Because R50 (*sym16*) was created via gamma radiation (Kneen and LaRue 1988), it is highly probable that it is a loss-of-function mutant, suggesting that the defect in R50 CK metabolism is probably one occurring in its degradation.

The ethylene-response phenotype of R50

Recently, a role for ethylene in the nodulation phenotype of R50 was established using ethylene inhibitors that restored nodulation to a level similar to that of 'Sparkle' (Guinel and Sloetjes 2000). An inhibitory role for ethylene in the regulation of nodule organogenesis had previously

Table 3. Concentration (in pmol·g^{-1} FM) of individual CKs identified by LC (+ES) MS/MS from shoots and roots of *Pisum sativum* 'Sparkle' and R50 at 9 DAP.

	'Sparkle'			R50		
CK	Shoot	Root	Total plant	Shoot	Root	Total plant
cis-Z	nd	nd	nd	nd	nd	nd
trans-Z	nd	nd	nd	nd	nd	nd
DZ	nd	nd	nd	nd	nd	nd
iP	0.26±0.10	0.67±0.23	0.98±0.13	0.19±0.11	0.27±0.06	0.45±0.12
Total free bases	0.26±0.10	0.67±0.23	0.98±0.13	0.19±0.11	0.27±0.06	0.45±0.12
cis-[9R]Z	0.44±0.07	2.27±0.85	2.77±0.84	0.53±0.34	1.03±0.26	1.57±0.43
trans-[9R]Z	0.66±0.15	2.17±0.02	2.92±0.13	0.66±0.55	2.32±0.53	2.98±0.97
[9R]DZ	0.44±0.28	0.18±0.15	1.11±0.12	1.23±0.77	2.03±0.76	4.21±1.16
[9R]iP	3.57±1.51	8.40±4.21	11.78±5.93	5.99±2.96	14.35±3.22	17.27±5.55
Total Ribosides	5.12±1.87	13.03±3.53	18.04±5.68	8.41±3.90	15.82±4.39	22.43±9.03
cis-[9R-MP]Z	4.00±0.76	3.67±0.79	9.40±1.10	6.81±2.18	3.53±0.83	10.35±2.96
trans-[9R-MP]Z	8.66±0.91	6.06±3.00	13.80±4.01	10.71±5.64	5.32±3.41	17.71±8.81
[9R-MP]DZ	10.11±4.59	5.97±2.00	20.68±4.75	6.97±2.51	12.13±3.85	15.36±5.69
iP-NT	2.51±0.21	3.92±0.31	6.35±0.52	4.89±1.62	4.71±0.24	9.59±1.17
Total nucleotides	17.69±6.01	13.60±6.01	28.29±9.29	23.54±12.56	28.03±5.75	50.86±16.22

Note: The following CKs and CK derivatives were analyzed: [9R]iP, isopentenyl-adenosine; *trans*- and *cis*-[9R]Z, *trans*- and *cis*-zeatin riboside; [9R]DZ, dihydrozeatin riboside; iP-NT, isopentenyl-nucleotide; *trans*- and *cis*-[9R-MP]Z; *trans*- and *cis*-zeatin nucleotide; [9R-MP]DZ, dihydrozeatin nucleotide; and iP, isopentenyl adenine. nd, not determined. Values are means ± SE (n = 3–4).

Table 4. Concentration (in pmol·g^{-1} FM) of individual CKs identified by LC (+ES) MS/MS from shoots and roots of *Pisum sativum* 'Sparkle' and R50 at 17 DAP.

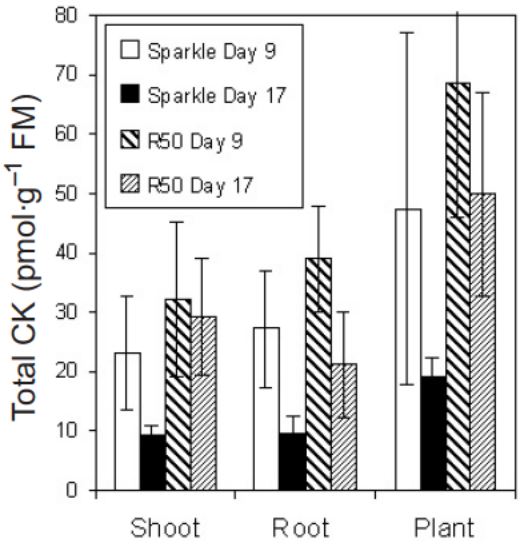
	'SparkleA'			R50		
CK	Shoot	Root	Total plant	Shoot	Root	Total plant
cis-Z	nd	nd	nd	nd	nd	nd
trans-Z	nd	nd	nd	nd	nd	nd
DZ	nd	nd	nd	nd	nd	nd
iP	0.15±0.06	0.15±0.05	0.31±0.07	0.44±0.19	0.34±0.15	0.78±0.34
Total free bases	0.15±0.06	0.15±0.05	0.31±0.07	0.44±0.19	0.34±0.15	0.78±0.34
cis-[9R]Z	0.76±0.35	0.66±0.23	1.42±0.48	2.01±1.24	2.43±0.40	4.44±1.38
trans-[9R]Z	0.02±0.02	0.42±0.33	0.44±0.34	1.69±0.86	0.59±0.22	2.82±0.92
[9R]DZ	0.76±0.35	0.66±0.22	1.42±0.48	8.27±7.41	0.86±0.28	9.12±6.66
[9R]iP	1.79±0.81	0.54±0.16	2.33±0.88	3.29±1.46	0.99±0.36	4.28±1.71
Total ribosides	3.30±1.09	3.85±1.50	7.15±2.21	15.25±10.49	4.87±0.87	20.13±11.22
cis-[9R-MP]Z	1.60±0.69	3.83±2.26	5.43±2.94	2.47±0.68	11.75±7.30	14.21±7.91
trans-[9R-MP]Z	0.92±0.26	0.94±0.35	1.86±0.59	2.90±0.76	2.29±0.79	5.19±1.15
[9R-MP]DZ	0.55±0.15	0.25±0.08	0.61±0.27	2.37±0.33	0.84±0.30	3.36±0.44
iP-NT	3.12±1.32	0.89±0.37	4.02±1.33	6.56±3.45	1.82±0.64	8.37±3.28
Total nucleotides	5.82±1.10	5.62±2.44	11.45±2.69	13.57±4.57	15.91±8.23	28.97±6.01

Note: The following CKs and CK derivatives were analyzed: [9R]iP, isopentenyl-adenosine; *trans*- and *cis*-[9R]Z, *trans*- and *cis*-zeatin riboside; [9R]DZ, dihydrozeatin riboside; iP-NT, isopentenyl-nucleotide; *trans*- and *cis*-[9R-MP]Z; *trans*- and *cis*-zeatin nucleotide; [9R-MP]DZ, dihydrozeatin nucleotide; and iP, isopentenyl adenine. nd, not determined. Values are means ± SE (n = 3–4).

been shown in pea (e.g., Lee and LaRue 1992) and *Medicago truncatula* (Penmetsa and Cook 1997). In fact, the hypernodulation phenotype of the *M. truncatula* mutant *sickle* is linked to ethylene insensitivity (Penmetsa and Cook 1997). Because R50 produces few if any nodules when inoculated with *Rhizobium leguminosarum* and its nodulation phenotype is restored with ethylene inhibitors (Guinel and Sloetjes 2000), it was thought that R50 overproduces ethylene or over-responds to endogenous concentrations of the hormone. However, we have been unable to differentiate 'Sparkle' from R50 on the basis of their endogenous production of ethylene (data not shown). Furthermore, we show here that the roots of both 'Sparkle' and R50 were qualitatively comparable in their response to exogenous ethylene, whereas the shoots of R50 seemed to be less sensitive to the hormone. Thus, R50 seedlings appeared to have neither overproduction of, nor oversensitivity to, ethylene. On the contrary, the shoots of R50, unlike the roots, were particularly insensitive to exogenous ethylene.

Many hormonal mutants with distinct organ phenotypes have been characterized. For example, the hypernodulating mutant of *M. truncatula*, *sunn*, exhibits root growth that is

Fig. 7. Total CK concentration in the shoot, root, and entire plant of *Pisum sativum* 'Sparkle' and R50 at 9 and 17 DAP. Values are means ± SE of three replicates.



insensitive to ethylene whereas its triple response phenotype appears normal, suggesting proper shoot sensitivity to the hormone (Penmetsa et al. 2003). Interestingly, the nodulation of *sunn* is sensitive to ethylene, suggesting that roots and nodules also respond differently to ethylene. In *Arabidopsis*, atypical ethylene phenotypes induced by mutations have been localized in the shoot (Larsen and Chang 2001) and root (Luschnig et al. 1998). Furthermore, using *wol/cre1* mutants, it was established that the cytokinin receptor CRE1 is predominantly located in the root, resulting in a specific root phenotype in the mutants (reviewed in Deruère and Kieber 2002). This disparity in organ responses to hormones has also been shown in transgenic CK-overproducing plants; for example, the stem vasculature of *Arabidopsis* (Rupp et al. 1999) or tobacco (Ainley et al. 1993) is increased and expanded, whereas the root vasculature appears normal. Moreover, a transgenic *Arabidopsis* line overexpressing the KNAT2 homeodomain protein exhibits some features of a CK overproducer, but the phenotype is restricted to the aerial parts of light-grown plants (Hamant et al. 2002). Werner et al. (2001) proposed that different organ responses to hormones could be due to distinct patterns of gene expression in hormone-target cells.

The role of ethylene and cytokinin in the R50 phenotype

Individual roles of ethylene and CK are often difficult to distinguish (Cary et al. 1995; Lorteau et al. 2001); therefore, it is possible that the two hormones are coupled in inducing some of the effects observed in R50. CKs are known to promote the production of ethylene in planta (e.g., Lorteau et al. 2001). In *Arabidopsis*, the ethylene-insensitive mutant *eti5* overproduces both CK (Kudryakova et al. 2001) and ethylene (Harpham et al. 1991). In addition, when transgenic *Arabidopsis* plants carrying the *Agrobacterium tumefaciens* CK-biosynthesis gene *ipt* are crossed with ethylene-insensitive mutants (including *eti5*), many of the phenotypic effects previously observed in the transgenic parent are shown to be the result of elevated ethylene levels (van der Graaff et al. 2001). For example, paleness of the leaves and short stature are two characteristics of the transgenic plants that are corrected upon crossing with the ethylene-insensitive mutants.

R50 also has pale leaves and a short stature and, coupled with the differences in the CK profiles, one would expect that the mutant would also produce more ethylene. The elevated CK concentrations could be responsible for the differences in ethylene response observed in roots and shoots of R50. The variations in the CK profile are intriguing as they are opposite to the elevated CK concentrations found in the roots of the *Arabidopsis* mutant *hoc1* (Catterou et al. 2002).

Conclusion

R50 appears to be the ideal tool to study the subtle roles played by endogenous ethylene and CKs in one of the most important agricultural symbiosis, i.e., nodulation. Among all the legume mutants that we are aware of, R50 is unique as it is a partial etiolation mutant that accumulates CKs in its shoots. This last characteristic could explain the differential response of shoots and roots towards ethylene.

Acknowledgements

The research was supported by Natural Sciences and Engineering Research Council of Canada operating grants to F.C.G. and R.J.N.E., and a Natural Sciences and Engineering Research Council of Canada Undergraduate Student Research Assistantship to E.M.W.; additional funding was provided to B.J.F. by Wilfrid Laurier University. We would like to thank J. Kent for some of the morphometric measurements and M.S. Allen for helping with the figures.

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