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The effects of resource availability and environmental conditions on genetic rankings for carbon isotope discrimination during growth in tomato and rice

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Abstract. Carbon isotope discrimination (Δ) is frequently used as an index of leaf intercellular CO_2 concentration (c_i) and variation in photosynthetic water use efficiency. In this study, the stability of Δ was evaluated in greenhouse-grown tomato and rice with respect to variable growth conditions including temperature, nutrient availability, soil flooding (in rice), irradiance, and root constriction in small soil volumes. Δ exhibited several characteristics indicative of contrasting set-point behaviour among genotypes of both crops. These included generally small main environmental effects and lower observed levels of genotype-by-environment interaction across the diverse treatments than observed in associated measures of relative growth rate, photosynthetic rate, biomass allocation pattern, or specific leaf area. Growth irradiance stood out among environmental parameters tested as having consistently large main affects on Δ for all genotypes screened in both crops. We suggest that this may be related to contrasting mechanisms of stomatal aperture modulation associated with the different environmental variables. For temperature and nutrient availability, feedback processes directly linked to c_i and / or metabolite pools associated with c_i may have played the primary role in coordinating stomatal conductance and photosynthetic capacity. In contrast, light has a direct effect on stomatal aperture in addition to feedback mediated through c_i.

Introduction

Plant photosynthetic water-use efficiency (WUE), defined here as the ratio of carbon assimilation (A) and transpiration (E) rates, is an important ecological and agronomic trait. It can affect plant performance not only when water is a limiting environmental resource, but also when the physiological costs of water uptake and transport limit stomatal opening (Comstock 2002; Sperry *et al*. 2002). Both natural plant populations and crop varieties show a considerable range of genetically heritable differences in WUE (Geber and Dawson 1997; Richards *et al*. 2002). These contrasts may reflect variable past selection for efficient resource use under water-limited conditions, contrasting cost / benefit relationships between WUE and plant capacities for water, carbon, and nutrient uptake in different genetic backgrounds, and / or weakly regulated differences resulting from independent factors affecting the separate behaviours of stomatal opening and the development of photosynthetic capacity. The lability of WUE under contrasting environmental conditions might also be affected by variation in the relative importance of these underlying factors.

Both A and E can be defined at the leaf surface as gaseous fluxes occurring through the same diffusion pathway via the stomatal pores. Due to the common pathway, the flux ratio is proportional to the ratio of the respective diffusion gradients between the surrounding atmosphere and intercellular leaf spaces:

$$WUE \equiv \frac{A}{E} = \frac{g_{CO_2}(c_a - c_i)}{g_{H_2O}(w_i - w_a)} = \frac{c_a - c_i}{1.6(w_i - w_a)}, \qquad (1)$$

where c and w refer to concentrations of CO_2 and H_2O, respectively, subscripts i and a refer to intercellular and

Abbreviations used: A, carbon assimilation rate; c_i, leaf intercellular CO_2 concentration; D, leaf-to-air vapour pressure gradient; E, transpiration rate; NUE, nitrogen-use efficiency; QTL, quantitative trait loci; RGR, relative growth rate; SLA, specific leaf area; WUE, water-use efficiency.

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ambient atmospheric pools, respectively, g_{CO_2} and g_{H_2O} are the stomatal conductances to CO_2 and H_2O, respectively, and 1.6 is the ratio of molecular diffusivity of the two gasses in air. The c_i is determined by photosynthetic physiology as discussed below, and w_i increases geometrically with leaf temperature.

Some broad limitations are implied by Eqn 1, in that WUE of the photosynthetic process can only be improved within the limitations of an effective diffusion gradient in CO_2. High WUE requires a low c_i, which contributes to a potential substrate limitation of the primary photosynthetic carboxylation reaction catalysed by RuBP carboxylase (Rubisco). This limitation results in a strong correlation between maximal stomatal conductance and maximum photosynthetic rate in plants (Korner *et al*. 1979; Wong *et al*. 1979). Owing to the high concentration of Rubisco in leaves, there is an expected trade-off between WUE and photosynthetic nitrogen-use efficiency (NUE), and a potential depression of A and growth if c_i is excessively low and nitrogen is limiting to growth. There are also potential advantages of high WUE to productivity, however, both when total water availability is limiting over the season, and when the costs of water transport within the plant are substantial. The magnitude of the difference between c_a and c_i is known to vary up to 3-fold among highly productive wild species and some crops, even under well-watered conditions (Turner 1993; Larcher 1995; Franks and Farquhar 1999; Bacon 2004).

The c_i is sometimes referred to as 'intrinsic' water-use efficiency. It is the parameter in Eqn 1 over which plants have the most direct control by modulating the co-regulation of stomatal conductance and carboxylation capacity (Eqn 2), and also reflects the potential trade-offs between WUE and NUE discussed above:

$$c_i = c_a - \frac{A}{g_{CO_2}}. \quad (2)$$

While crop-level water-use efficiency may also be influenced by both field conditions and management practices outside the scope of Eqn 1, c_i nonetheless represents many of the most important contributions from selectable plant traits towards variation in crop WUE (Bacon 2004).

A useful proxy to allow high-throughput screening for intrinsic WUE in C_3 plants is variation in carbon isotope discrimination (Δ). Atmospheric carbon dioxide is naturally composed of two stable isotopes, ^{13}C and ^{12}C. During photosynthesis, the lighter isotope is taken up at slightly faster rates, leading to a process of isotopic discrimination. For C_3 plants, the change in the abundance ratio of $^{13}C / ^{12}C$ between plant biomass and the atmospheric carbon source generally follows the simplified relationship:

$$\Delta = a + (b - a)\frac{c_i}{c_a}, \quad (3)$$

where a and b are discrimination constants associated with CO_2 diffusion in air and the carboxylation reactions in the leaf, respectively, with values of 4.4 and 27 per mil (‰), respectively. The dependency of Δ on the c_i / c_a ratio leads to its expected negative correlation with WUE (Farquhar *et al*. 1989). There is extensive variation in Δ among C_3 plant genotypes (Brugnoli and Farquhar 1999). While several potential factors can contribute to this, the most dominant source of variation is the ratio of stomatal conductance relative to internal photosynthetic capacity, which determines c_i. Δ is low when diffusion is more rate limiting, and high when carboxylation capacity is more limiting.

Δ is known to vary at times with environmental conditions as well as genetic differences. For example, Δ tends to be lower at high light (Ehleringer *et al*. 1986; Hanba *et al*. 1997; Carelli *et al*. 1999), low relative humidity (Madhavan *et al*. 1991; Barbour and Farquhar 2000; Sanchez Diaz *et al*. 2002), and under drought conditions (Cabuslay *et al*. 2002), and may show variable responses to factors such as nutrient conditions and temperature (Morecroft and Woodward 1996; Craufurd *et al*. 1999; Brück *et al.* 2001; Guo *et al*. 2002; Hamerlynck *et al*. 2004). Nonetheless, environmental factors often show consistent effects across a wide array of genotypes, and numerous studies have reported non-significant or low genotype-by-environment interactions (G × E) in field trials that include multiple year, plot and watering treatments (Hubick *et al*. 1988; Hall *et al*. 1994; Le Roux *et al*. 1996; Livingston *et al*. 1999; Merah *et al*. 1999; Pennington *et al*. 1999). Only a few studies have found larger G × E (Cregg *et al*. 2000; Ponton *et al*. 2002). In field-grown barley, Teulat *et al*. (2002) described ten significant quantitative trait loci (QTL) contributing to variation in Δ. Over half of the QTL exhibited main effects across multiple environments, two had effects in only one environment, and only two showed QTL by environment interaction.

Here we test the hypothesis that Δ primarily reflects an intrinsic set-point in the co-regulation of stomatal conductance and carbon assimilation. This hypothesis predicts that (1) genetic rankings for Δ should show minimal G × E interaction regardless of the nature of environmental perturbation, and (2) this should contrast with underlying characters of carboxylation capacity, leaf morphology, and stomatal behaviour, which may show genotype-specific patterns of complementary environmental response resulting in stable Δ. We focused on factors that could dramatically affect the regulatory balance between carboxylation capacity and stomatal conductance in well-watered plants but we excluded stress responses to drought or extreme conditions. In particular, we evaluated responses to (1) growth temperature, which has effects on carboxylation capacity above and below the photosynthetic temperature optimum as well as geometrically increasing transpiration potential with increasing leaf temperature, (2) nutrient availability, which affects carboxylation capacity through leaf

protein content but has no inherent effect on *E*, (3) irradiance level, which affects electron-transport-limited carboxylation rates, and (4) soil conditions such as medium and water saturation. Since this study was conducted in greenhouses, we also evaluated the response to soil volume / root constriction and the interactions of soil volume with plant size and age.

We chose to study two crop systems, tomato (*Solanum lycopersicum* L.) and rice (*Oryza sativa* L.). The two crops share numerous strengths as experimental systems, including well-developed genomics tools (Budiman *et al*. 2000; Goff *et al*. 2002; Van der Hoeven *et al*. 2002) and a history of work in water-relations (Martin *et al*. 1989; Dingkuhn *et al*. 1991*a*; Martin *et al*. 1999; Stiller *et al*. 2003), but also provide strong contrasts. These include phylogenetic divergence (eudicot *v.* monocot) and an annual desert *v.* a perennial wetland ancestor for tomato and rice, respectively. There are well-characterised, permanent mapping populations derived from crosses between cultivated tomato and related wild species of *Solanum* (Eshed and Zamir 1994; Monforte and Tanksley 2000), some of which are known from past studies to express higher WUE than the cultivated tomato (Martin *et al*. 1989). Rice shows tremendous ecological amplitude in both temperate and tropical climates with adaptation to a wide range of cultivation conditions, from flooded soil and paddies to dry upland fields. Genetic tools and stable mapping populations for exploring these contrasts are readily available (Huang *et al*. 1997; Lin *et al*. 1998; Ishimaru *et al*. 2001).

Materials and methods

Plant materials

Tomato genotypes included two cultivars, *Solanum lycopersicum* (L.) E6203 and *S. lycopersicum* (L.) M82, and single genotypes of two related wild species, *S. pennellii* (Correll) D'Arcy accession LA716 and *S. habrochaites* (Knapp & Spooner) accession LA1777. Rice genotypes included several cultivars of *Oryza sativa* L, Nipponbare (temperate japonica), Azucena (tropical japonica), Jefferson (tropical japonica), Kasalath (aus), Teqing (indica), and IR64 (indica), as well as one accession of the putative wild ancestor, *O. rufipogon* (Griff.) (*IRGC#105491*).

Growth conditions

All plants were grown in a glasshouse environment with controlled temperature, humidity, and supplemental high intensity discharge (HID) lighting (a bank of alternating 1000-W Na-vapour and Me-Halide lamps) at the Boyce Thompson Institute for Plant Research on the Cornell University campus in Ithaca, New York. Plants were monitored during growth for mean photosynthetically active radiation (400–700 nm; PAR), temperature, humidity, and ambient [CO_2] within each greenhouse bay. Data were collected every 30 s throughout plant growth and averaged in 30-min intervals by a CR10 Datalogger (Campbell Scientific, Logan, UT). Greenhouse air samples from different greenhouse bays were pumped in repeating sequence to a single Infrared gas analyser (Li-Cor Gas-Hound, Li-Cor, Lincoln, NE) for assessment of [CO_2]. The analyser cycled through a full set of comparative samples from each greenhouse every 3 min. These readings were accumulated by the same datalogger system mentioned above and converted to independent averages of each greenhouse at 30-min intervals. Monitored environmental data is given for each experiment in Table 1.

Irradiance

Attempts were made to standardise total irradiance levels across experiments. However, natural sunlight differed dramatically seasonally, and some variation in combined totals was observed despite compensatory adjustments in HID lamp arrangement (Table 1). Values given (Table 1) are total photoperiod averages and include considerable diurnal variation. Typical irradiance was 500–600 $\mu mol\, m^{-2}\, s^{-1}$ PAR during the earliest morning and latest evening hours (largely from HID sources) and above 1000 $\mu mol\, m^{-2}\, s^{-1}$ PAR at midday. This ensured 90% or greater photosynthetic light saturation for a substantial portion of each day. Where variation in PAR is presented as an experimental treatment it represents variation in HID intensity.

Soil volume

Unless otherwise indicated, plants were grown to measurement age at 3–4 weeks past germination in 0.14-m diameter pots with 2.5 L soil volume. Two reported experiments, however, specifically explore variation in soil volume associated with pot size. The study reported in the section on 'Plant age and soil volume' used pot diameters of 0.05, 0.07, 0.14, 0.25 and 0.36 m, with soil volumes of 0.33, 0.30, 2.5, 20.0, and 35.0 L, respectively. The two smallest volumes contrasted a tall,

Table 1. Environmental conditions during growth at the BTI greenhouses

Treatment variables in the first column correspond to subheadings in the Results section and respective plantings discussed therein. PP, photoperiod; PAR, photosynthetically active radiation averaged over the photoperiod and including natural sunlight and supplemental HID lamps; RH, relative humidity; Airtemp is measured at plant height; [CO_2], average photoperiod carbon dioxide concentration

Experiment	Crop	PP (h)	PAR ($\mu mol\, m^{-2}\, s^{-1}$)	RH (%)	Airtemp (°C)	[CO_2] ($\mu L\, L^{-1}$)
Temperature	Both	14	625	50	variable	380
Nutrient	Tomato	14	625	50	28	380
	Rice	14	800	60	28	341
Soil volume × nutrient	Both	14	800	60	28	341
Soil volume × age	Rice	14	640	48	28	375
Irradiance	Tomato	14	variable	48	28	375
	Rice	12	variable	45	30	380

narrow 'cone-tainer' with a shorter and broader pot differing greatly in width-to-depth ratio, but not in volume. Data presented on the interaction of soil volume and fertiliser were collected from plants grown in pots with diameters of 0.07, 0.08, and 0.14 m and soil volumes of 0.3, 0.8, and 2.5 L, respectively.

Soil media and fertilisation

Three potting soil mixes were used: (1) mineral soil mix: 3 : 1 : 1 fritted clay : sand : topsoil (screened and pasteurised) with dolomitic lime, gypsum, superphosphate, and a micronutrient supplement (Micromax Plus, Scotts Co., Marysville, OH) added as amendments at rates of 2.7, 4.5, 1.1, and 0.85 kg m^{-3}, respectively. This mix could be fully washed from root systems and was used whenever root harvests were planned. (2) Tomato mix: 2 : 1 : 1 : 1 vermiculite : peat : sand : perlite with dolomitic lime and micronutrient supplement (Unimix III, Scotts Co.) added as amendments at rates of 2.7 and 0.64 kg m^{-3}, respectively. (3) Rice mix: 6 : 3 : 3 : 1 : 1 vermiculite : peat : fritted clay : sand : topsoil and with dolomitic lime, gypsum, superphosphate, and two micronutrient supplements (Unimix III and Micromax Plus) added as amendments at rates of 3.9, 1.5, 0.38, 0.71 and 0.28 kg m^{-3}, respectively.

Tomatoes were grown at all times in a manner fostering fully aerobic soil conditions. Rice was grown with two soil management regimes, one with well drained aerobic soil watered daily or as needed to prevent moisture stress, and another with flooded, anaerobic soil conditions generated by submerging the bottom 25% of the soil profile (18 cm total height) in trays of standing water (usually four 1.1-L pots per tray). The wicking of moisture in these short soil profiles resulted in saturated conditions up to the soil surface. Unless otherwise noted, plants were fertilised once every other day beginning one week after germination. Tomatoes received Peters Excel 15 : 5 : 15 at a concentration providing 100 μg g^{-1} N, and rice with Peters 20 : 10 : 20 and an iron chelate (Sprint 330, Becker Underwood Inc., Ames, IA) applied at a concentration giving 100 μg g^{-1} available nitrogen and 0.45 g L^{-1} chelate.

Isotopic analyses

Δ was evaluated at the Cornell Stable Isotope Laboratory (COIL) with a Finnigan Matt Delta Plus isotope ratio mass spectrometer (IRMS). Isotope ratio data were provided by COIL relative to the IAEA standard PDB, as:

$$\delta^{13}C = \left(\frac{^{13}C/^{12}C \text{ sample}}{^{13}C/^{12}C \text{ standard}} - 1 \right) \times 1000, \text{‰}. \quad (4)$$

$\delta^{13}C$ was measured for plant samples, and Δ was calculated as (Farquhar and Richards 1984):

$$\Delta = \frac{\delta^{13}C_{air} - \delta^{13}C_{plant}}{1 + \delta^{13}C_{plant}/1000}, \text{‰}. \quad (5)$$

The $\delta^{13}C$ of atmospheric CO_2 was measured directly only at the beginning of the project to establish a relationship between $\delta^{13}C_{air}$ and 1 / [CO_2] (Keeling 1958) in the growth facility. In each experiment reported here, atmospheric [CO_2] was measured continuously in each greenhouse bay throughout the growth interval, and mean daytime [CO_2] from the week preceding sampling was converted to an estimate of $\delta^{13}C_{air}$ by:

$$\delta^{13}C_{air} = 5273 \times \frac{1}{[CO_2]} - 22.94, \text{‰}. \quad (6)$$

Leaves for isotopic analyses were chosen from the youngest cohort of leaves that had completed the phase of rapid expansion. These usually represented the largest leaves on the young vegetative plants, and occupied upper canopy positions experiencing maximal illumination. Unless otherwise indicated, samples consisted of several leaflets taken from two such leaves for each tomato plant and two full leaf blades from each of two or more tillers per rice plant. Where specific leaf area is reported, it was measured from fresh-leaf projected area and total dry weight on these isotope sample leaves. After drying for 48 h at 60°C, leaf samples were ground into an homogeneous powder and 2-mg subsamples weighed for isotopic analysis. In addition to $\delta^{13}C$, COIL analyses provided elemental composition in percent N (%N) and percent C (%C), with measurement precisions of ± 0.1% and ± 0.75%, respectively.

Statistics

Most data reported were analysed by two- or three-level analysis of variance (ANOVA) encompassing genotype and one or more growth conditions (i.e. nutrient level, irradiance level, temperature) in a factorial design. Sample sizes, unless otherwise stated, were six plants per genotype–treatment combination. For isotopic analyses, the six plants were usually bulked into three samples, each representing two plants, for economy. Precision (one standard deviation, or s.d.) of repeated $\delta^{13}C$ measures on a single ground plant sample was generally ≤ 0.12‰, while the s.d. for a given genotype / treatment averaged 0.35‰ for tomato and 0.2‰ for rice. Bulked samples were therefore approximate representations of the mean values of the contributing plants, although there was some loss of statistical power owing to reduced replication. Some larger plantings incorporated a randomised block design of three or six blocks. Blocks were always arranged along a north–south axis that allowed assessment of influences related to the greenhouse air-handling system. Though well mixed by fan networks within each bay, make-up air was added only at the north end of each greenhouse and resulted in modest gradients in temperature, relative humidity (RH), and possibly other factors, within the bay. Block effects were generally significant but small and consistently more important for some measured plant parameters than others. Most influenced were %N and SLA, and least influenced were Δ and measures related to plant height and leaf dimensions. Prior to some plantings, variation in HID lamp output was measured at each plant's future position, and these values, when collected, were entered into final statistical analyses as a covariate. Reported temperature treatments refer to sets of plants in three contrasting greenhouse bays and therefore have an element of pseudoreplication. The bays were individually monitored to document consistency of light, RH, [CO_2] and air temperature.

Results and discussion

Several different genotypes were used in the following screens as specified within each section below. The genotypes were chosen to maximise differences in Δ, as observed in preliminary screens, and also because they represent parental lines of mapping populations that can be used for the genetic dissection of the traits studied here. Except where otherwise noted, plants were grown in 2.5-L containers and sampled at 3–4 weeks from germination.

Δ was measured in all experiments reported below. Several additional measurements were also included in several experiments to give greater insight into the responses of component processes affecting Δ. (1) Relative growth rates (RGR) from germination to harvest show overall plant carbon gain, integrating the effects of availability of resources such as light and nutrients, the physiological state as affected by temperature, and Δ itself. (2) Percent root and leaf biomass represent allocation patterns of biomass investment on a dry weight basis. Percent root was only

measured on plants grown in the mineral soil media (see Materials and methods). For consistency across experiments and conditions, percent leaf refers to leaf blade as a fraction of total shoot rather than total plant biomass. These allocation patterns have implications for nutrient acquisition and water transport capacities relative to the demands of photosynthetic tissues. (3) Specific leaf area (SLA, $m^2\ kg^{-1}$) reflects the amount of leaf surface area available for diffusive exchange of CO_2 relative to the potential carboxylation capacity of the leaf. (4) Photosynthetic carbon assimilation rates (A) were measured with a portable gas-exchange system (Li-Cor 6400) and are expressed relativised to both unit leaf area ($\mu mol\ m^{-2}\ s^{-1}$) and unit leaf biomass ($\mu mol\ kg^{-1}\ s^{-1}$). Expressing photosynthesis per unit leaf area links carboxylation and diffusion steps in a manner most directly related to the determination of Δ (Eqn 2), while the mass-based expression is more directly relevant to resource investment and RGR. (5) Percent N was measured on bulk leaf tissue. Leaves compose the largest single biomass fraction in these young vegetative plants and this fraction is expected to have higher nitrogen content than others; thus, %N of the leaf fraction is a good index of total plant nitrogen status.

Temperature effects

Growth temperature responses were tested over the range 23–33°C for mean daytime values. This spans the photosynthetic temperature optimum of warm season C_3 crops and includes typical growth temperatures for both crops used here under favourable conditions. Responses differed substantially between tomato and rice (Fig. 1; Tables 2, 3).

Tomato

RGR was substantially higher for tomato than for rice in these early vegetative stages, and somewhat less sensitive to temperature (Fig. 1*A*, *B*). The wild species, *S. habrochaites* and *S. pennellii*, had higher RGR than the tomato cultivars and there was a distinct temperature optimum for growth at or near 28°C with significant declines at both extreme temperatures. SLA was almost unaffected by temperature (Fig. 1*E*). Photosynthetic rates in both area (Fig. 1*G*) and mass based units (Fig. 1*I*) were highest at the highest temperature, but did not vary as greatly overall as in rice. The wild species tended to have higher A than the cultivars, particularly at low temperatures or when expressed per unit leaf mass. Plants at high temperatures were taller and had significantly greater biomass in stems (data not shown), such that, despite declining %root, %leaf also declined with increasing temperature (Fig. 1*C*). Temperature had little effect on Δ in tomato, with only a small trend towards higher values at higher temperature (Fig. 1*K*). A significant genotype × soil type interaction was seen, however, owing to the behaviour of *S. pennellii*, which had substantially higher Δ in mineral soil than in the peat and vermiculite based 'tomato mix' (Table 2, see also Fig. 2*L*, *M*).

Rice

All rice varieties showed dramatic depression in RGR at the lowest temperature and no change in RGR across the two higher temperature treatments (Fig. 1*B*). There were no significant differences in RGR responses to temperature among the five genotypes tested nor between flooded *v.* aerated soil conditions (Table 3). A, expressed on an area basis, was actually highest at the lowest temperature for all rice varieties except Teqing (Fig. 1*H*). These high rates were associated with thicker leaves (low SLA; Fig. 1*F*). Expressed on a mass basis, A was highest at the intermediate temperature, 28°C (Fig. 1*J*). Δ showed strong genetic differences and very stable rankings with no significant G × E interactions (Table 3). These stable rankings contrast strongly with several other parameters such as SLA, A, allocation to productive leaf tissue (Fig. 1*D*), and percent carbon content of the leaves (data not shown), all of which showed responses that differed dramatically among different genotypes and / or soil conditions (Table 3).

Stomatal response to temperature is influenced both by the temperature sensitivity of carboxylation capacity in the leaf and by the increased transpiration rates which can be associated with large leaf-to-air vapour pressure gradients (D) at high temperatures. If temperature is increased while holding absolute humidity constant (i.e. allowing D to increase), the net effect is generally one of stomatal closure. In contrast, if relative humidity is held constant, stomatal conductance will either be constant or increase in proportion to photosynthetic capacity (Ball *et al*. 1987; Matzner and Comstock 2001). In our studies, relative humidity was held constant at 50% and the range of temperatures bracketed the expected optimum for C_3 photosynthesis. Even over this limited range, the more tropical origins of rice are apparent in its much more severe growth depression at 23°C compared with tomato. Rice also showed more obvious leaf acclimation responses during growth, altering SLA and the relationships between area and mass-based photosynthesis. The small shift observed in Δ with increased temperature in both crops could be related to several factors, including transpirational cooling at supra-optimal temperatures, depression of carboxylation efficiency above the temperature optimum, and possible additional isotope effects associated with increased respiration at high temperature (Ghashghaie *et al*. 2003). Other controlled-temperature studies have also reported a positive correlation between temperature and Δ (Morecroft and Woodward 1996). But, most importantly, the shift in Δ was consistent in magnitude across genotypes in both rice and tomato.

Δ is not directly proportional to WUE across temperature treatments because of large increases in w_i affecting the denominator of Eqn 1 as leaf temperature increases.

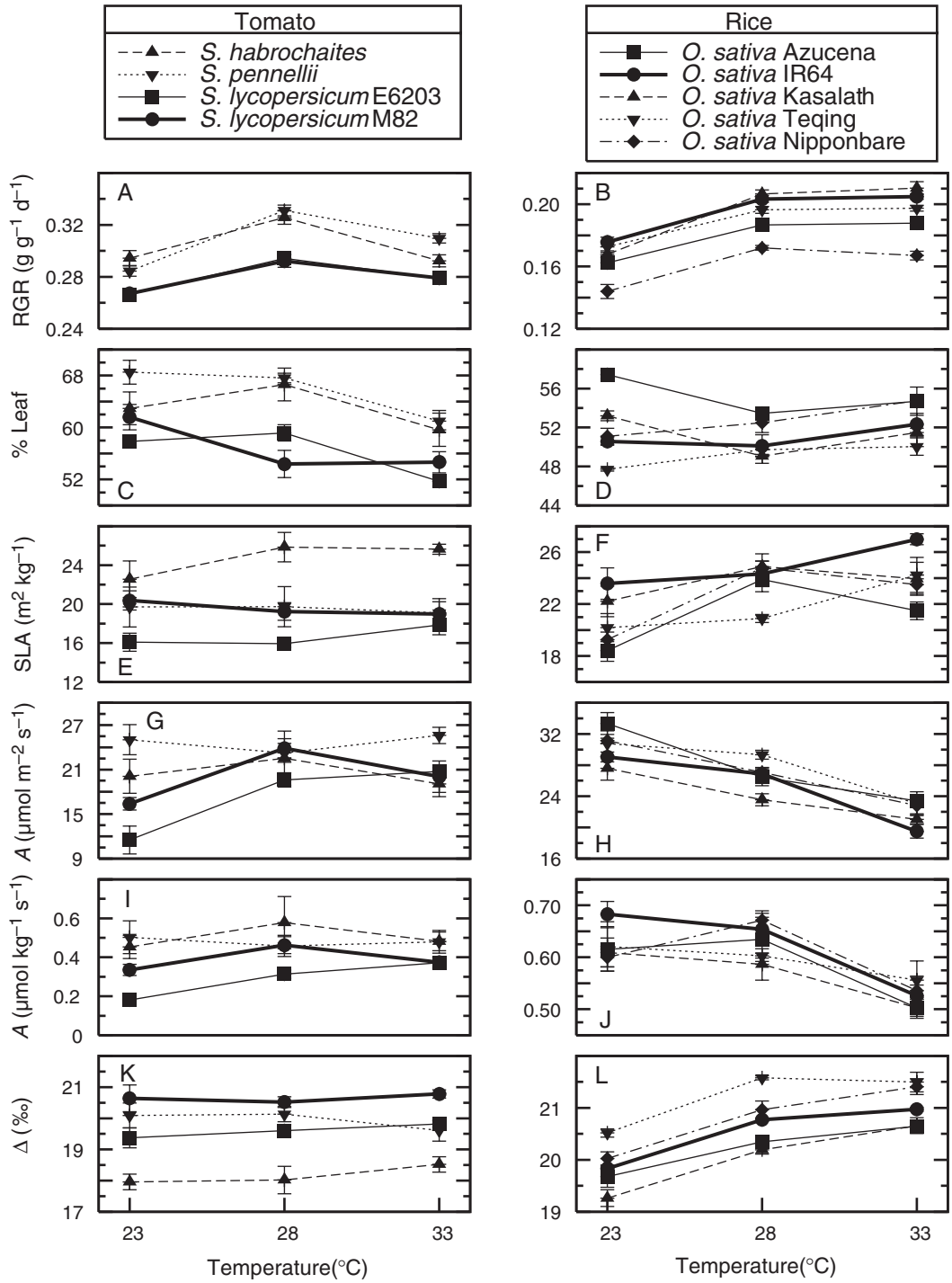

Fig. 1. Effects of variation in mean growth temperature during the photoperiod for a selection of both tomato (panels *A*, *C*, *E*, *G*, *I*, *K*) and rice (panels *B*, *D*, *F*, *H*, *J*, *L*) genotypes. These panels represent half of the data (one soil medium) dealt with in factorial design in Tables 2, 3. Shown are the relative growth rates (*A*, *B*), percent of shoot biomass allocated to leaf blade tissues (*C*, *D*), leaf area per unit leaf mass (*E*, *F*), photosynthetic carbon assimilation per unit leaf area per second (*G*, *H*), the same carbon assimilation rate expressed per unit leaf mass per second (*I*, *J*), and carbon isotope discrimination (*K*, *L*). Each data point represents six plants for RGR, %leaf, and Δ, and four plants for SLA and *A*. Error bars show ± one SE. Plot symbols represent different genotypes. In tomato panels, wild species relatives are also distinguished from tomato cultivars by dashed and solid lines, respectively. In rice panels, line dashing is a visual aid but follows no specific pattern.

Table 2. Effects on tomato of three growth temperatures and two soil conditions in factorial design

This table evaluates the stability of genetic ranking of four tomato genotypes for Δ and a variety of physiological and morphological variables. Genotypes include *S. lycopersicum* M82, *S. lycopersicum* E6203, *S. pennellii* and *S. habrochaites*. Soil conditions contrast a 'standard tomato-mix' (Fig. 1*A*, *C*, *E*, *G*, *I*, *K*) with a 'mineral' potting soil (not shown graphically). $n = 6$ plants per genotype × treatment combination. All plants were measured individually for RGR, only four of the six for %leaf (relative to above ground biomass), and *A*, and three bulked samples of two plants each for %N, and Δ. The total degrees of freedom (df) are given for each variable in the second column, and the degrees of freedom for each main treatment and interaction terms (constant for all measures) are given in the column headings. RGR is based on seed and final shoot biomass, SLA is m^{-2} leaf area, kg^{-1} dry weight, *A* is net photosynthetic rate expressed first per unit leaf area and again per unit leaf biomass, and %N and Δ are based on bulk dried leaf blade tissue. The table gives sums of squares (SS) for each model effect and *P* values; ****, $P<0.0001$; ***, 0.001; **, 0.01, and *, 0.05; ns, not significant

Parameter	df		Model	Error	Genotype df = 3	Day °C df = 2	Soil df = 1	G × °C df = 6	G × S df = 3	S × °C df = 2	G × °C × S df = 6
RGR ($g\,g^{-1}\,day^{-1}$)	143	SS	0.0601	0.0131	0.0279	0.0268	0.0009	0.0033	0.0003	0.0003	0.0005
		P	****		****	****	***	****	ns	ns	ns
%leaf	91	SS	0.2933	0.0683	0.1806	0.0545	0.0321	0.0122	0.0072	0.0005	0.0172
		P	****		****	****	****	ns	ns	ns	*
%root	70	SS	0.1114	0.0236	0.0797	0.0220	n/a	0.0120	n/a	n/a	n/a
		P	****		****	****	n/a	***	n/a	n/a	n/a
SLA ($m^2\,kg^{-1}$)	85	SS	869.5	432.4	590.6	55.0	4.6	28.5	87.4	22.2	58.0
		P	****		****	*	ns	ns	0.0137	ns	ns
A ($\mu mol\,m^{-2}\,s^{-1}$)	82	SS	996.7	573.0	466.2	163.6	9.6	158.7	7.4	75.4	81.7
		P	****		****	***	ns	*	ns	*	ns
A ($kg\,m^{-2}\,s^{-1}$)	82	SS	0.8439	0.5021	0.4300	0.1377	0.0012	0.0739	0.0321	0.0717	0.0592
		P	****		****	***	ns	ns	ns	*	ns
%N	71	SS	49.90	10.46	42.78	3.80	0.06	0.81	0.40	1.49	0.56
		P	****		****	***	ns	ns	ns	0.04	ns
Δ (‰)	71	SS	67.93	10.32	56.00	1.71	3.83	2.34	3.06	0.52	0.48
		P	****		****	*	****	ns	**	ns	ns

Table 3. Effects on rice of three growth temperatures and two soil conditions in factorial design

The ranking stability of five rice genotypes is evaluated for Δ and a variety of physiological and morphological variables. All genotypes are cultivars of *O. sativa* and include Azucena, IR64, Nipponbare, Kasalath, and Teqing. The two soil conditions are both standard 'rice-mix' media but under contrasting aerobic (Fig. 1*B*, *D*, *F*, *H*, *J*, *L*) *v.* flooded conditions (data not shown graphically). $n = 6$ plants per genotype by treatment combination. Sample sizes, variable, column, and row definitions are similar to Table 2

Parameter	df		Model	Error	Genotype df = 4	Day °C df = 2	Soil df = 1	G × °C df = 8	G × S df = 4	S × °C df = 2	G × °C × S df = 8
RGR ($g\,g^{-1}\,day^{-1}$)	179	SS	0.0700	0.0080	0.0318	0.0359	0.0006	0.0007	0.0002	0.0001	0.0006
		P	****		****	****	***	ns	ns	ns	ns
%leaf	141	SS	0.0814	0.0303	0.0497	0.0041	0.0033	0.0175	0.0023	0.0042	0.0024
		P	****		****	***	***	****	ns	***	ns
SLA ($m^2\,kg^{-1}$)	141	SS	806.1	487.0	251.2	345.0	2.0	140.2	2.7	3.7	61.2
		P	****		****	****	ns	***	ns	ns	ns
A ($\mu mol\,m^{-2}\,s^{-1}$)	141	SS	1230.8	556.0	201.9	508.7	6.2	97.4	118.2	255.1	41.9
		P	****		****	****	ns	*	***	****	ns
A ($kg\,m^{-2}\,s^{-1}$)	141	SS	0.574	0.5269	0.1706	0.1043	0.0155	0.0401	0.0668	0.1625	0.0217
		P	****		****	****	ns	ns	**	****	ns
%N	89	SS	20.725	2.736	8.361	10.705	0.002	0.611	0.103	0.404	0.539
		P	****		****	****	ns	ns	ns	*	ns
Δ (‰)	89	SS	44.39	3.35	15.13	26.43	1.42	0.47	0.36	0.17	0.40
		P	****		****	****	****	ns	ns	ns	ns

Nonetheless, the ranking of plants for WUE by Δ still holds within each treatment, and the lack of G × E for Δ implies a similar result for WUE, although the magnitudes of the main treatment effects would be much greater for WUE than for Δ.

Nutrient treatments

While nutrient levels were adjusted by application of balanced fertiliser, for convenience we refer only to the level of nitrogen in each treatment. Nitrogen is also the nutrient most often discussed in the context of WUE because of

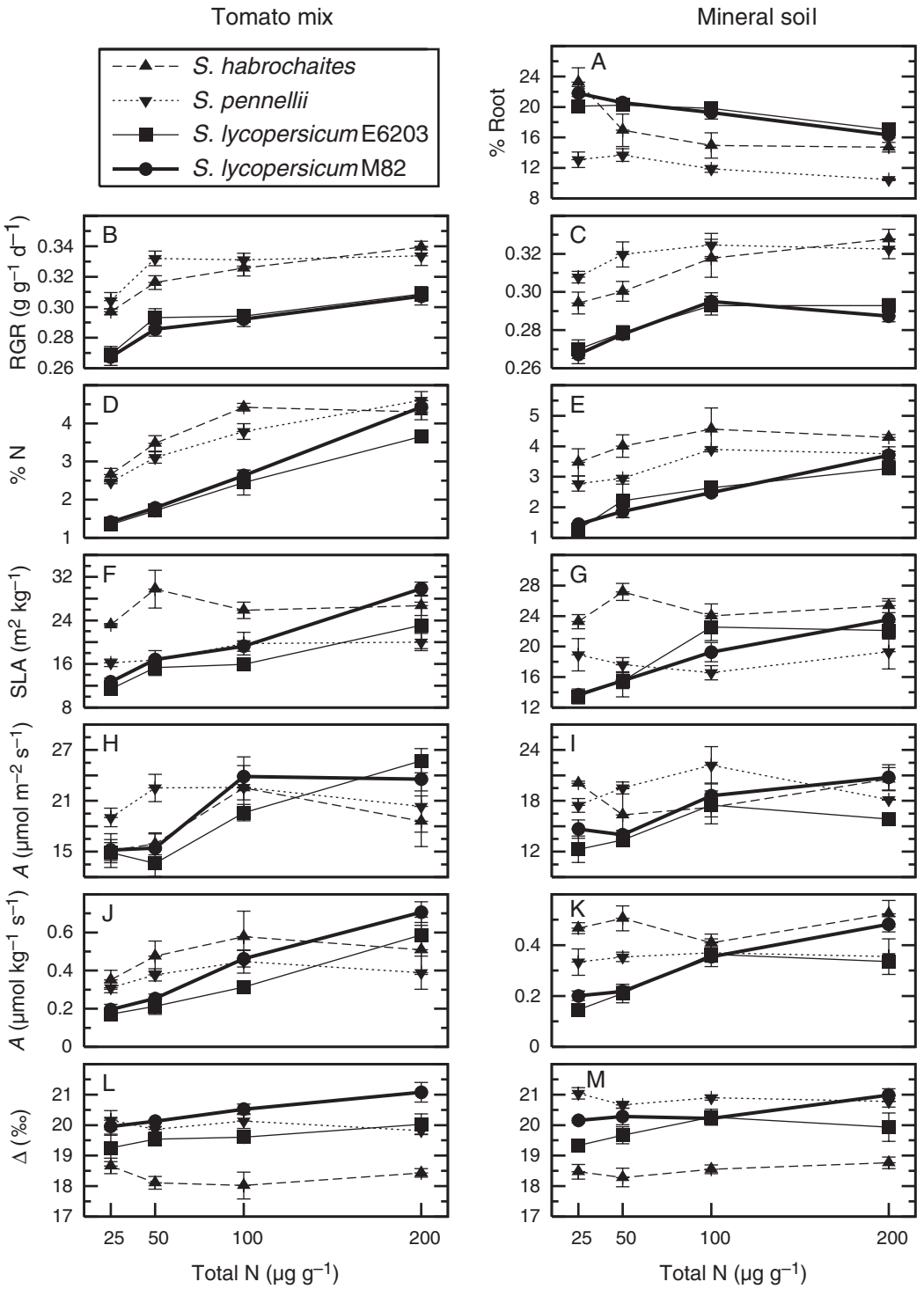

Fig. 2. Effects of nutrient level and soil medium on tomato. Two potting media were tested factorially with the nutrient levels, (1) a mineral soil (panels *A*, *C*, *E*, *G*, *I*, *K*, *M*) and (2) a standard vermiculite / peat-based soil: 'tomato-mix' (panels *B*, *D*, *F*, *H*, *J*, *L*). Shown are percent root, calculated on soil-washing in the mineral soil only and representing all size classes of roots (*A*), the relative growth rates (*B*, *C*), %N measured for leaf-blade tissue only (*D*, *E*), leaf area per unit leaf mass (*F*, *G*), photosynthetic carbon assimilation per unit leaf area per second (*H*, *I*), the same carbon assimilation expressed as a rate per unit leaf mass per second (*J*, *K*), and Δ (*L*, *M*). Each data point represents the mean of six replicate plants for %root, RGR, %N, and Δ, and four plants for SLA and *A*. Error bars are ± one SE. Wild species relatives are also distinguished from tomato cultivars by dashed and solid lines, respectively.

the expected trade-offs between WUE and NUE mediated through c_i.

Tomato

Variation in nutrient availability had a large impact on the growth rate and allocation pattern in tomato and there were dramatic differences between the responses of the wild and cultivated species (Fig. 2; Table 4). For all species, growth rates increased substantially as fertiliser levels were increased from 25 to 100 μg (N) L^{-1} but tended to saturate at the highest nutrient level (Fig. 2*B*, *C*). The two tomato cultivars had a 2-fold increase in SLA (Fig. 2*F*, *G*) and 3-fold increases in %N (Fig. 2*D*, *E*) at high nutrient availability. Even if expressed on an area basis (not plotted), N content was substantially higher under high fertiliser despite much thinner leaves. These patterns were reflected in photosynthetic rates, which were higher at high nutrient availability and varied more on a mass basis (Fig. 2*J*, *K*) than on an area basis. *A* in the wild species showed similar qualitative responses to nutrient levels but of much smaller amplitude. *S. pennelli* increased in SLA by only 20% and *S. habrochaites*, which had the highest SLA overall, did not significantly vary in SLA among treatments. Percent leaf nitrogen increased only 40 and 60% in *S. pennellii* and *S. habrochaites*, respectively, and variation in photosynthetic rate was similarly modest in comparison to the domestic cultivar responses. On average, there was an approximately 25% reduction between extreme low and high nutrient treatments in allocation of biomass to roots (Fig. 2*A*). In contrast to its relatively constant leaf characteristics, *S. habrochaites* was distinguished by a 40% increase in % root in the lowest nutrient treatment, a significantly larger shift than other genotypes. Variation in Δ was primarily associated with genotype alone and much less so with soil and nutrient availability (Table 4; Fig. 2*L*, *M*). A very small genotype × nutrient level interaction was related to slightly larger increases in Δ at high nutrient levels for the cultivars than the wild species.

Rice

Rice was evaluated for nutrient treatments spanning 25–200 μg (N) L^{-1} using one cultivar, *O. sativa* Jefferson and the wild species *O. rufipogon* (Fig. 3). Fewer parameters were measured, but, relative to tomato, rice had less of a tendency towards a saturated growth response at high nutrient levels (Fig. 3*A*). Leaf nitrogen contents were also less variable than in tomato (Fig. 3*B*). *O. rufipogon* had an increase in nitrogen content of 60% between the most extreme nutrient treatments while Jefferson showed no significant variation. Δ for rice was again primarily determined by genotype (Fig. 3*C*). There was a small effect of nutrient level and no significant genotype × nutrient level interaction. Interestingly, while high nutrient levels caused a consistently small increase in Δ in all tomato genotypes examined, a slight decrease was seen in both rice genotypes.

Table 4. Effects on tomato of four nutrient levels and two soil conditions in factorial design

The ranking stability of four tomato genotypes is evaluated for Δ and a variety of physiological and morphological variables. Genotypes include *S. lycopersicum* M82 and *S. lycopersicum* E6203, *S. pennellii* and *S. habrochaites*. Soil conditions contrast a 'standard tomato-mix' (Fig. 2*B*, *D*, *F*, *H*, *J*, *L*) with a 'mineral' potting soil (Fig. 2*A*, *C*, *E*, *G*, *I*, *K*, *M*). $n = 6$ plants per genotype by treatment combination. Sample sizes, variable, column, and row definitions are similar to Table 2

Parameter	df		Model	Error	Genotype df = 3	Soil df = 2	Nutrient df = 1	G × S df = 6	G × N df = 3	S × N df = 2	G × S × N df = 6
RGR (g g^{-1} day^{-1})	189	SS	0.0831	0.0244	0.0503	0.0027	0.0235	0.0001	0.0018	0.0018	0.0005
		P	****		****	****	****	ns	ns	*	ns
SLA (m^2 kg^{-1})	112	SS	2851	726	1168	4	736	55	460	43	82
		P	****		****	ns	****	ns	****	ns	ns
%root	54	SS	0.0885	0.0175	0.0549	n/a	0.0186	n/a	0.0097	n/a	n/a
		P	****		****	n/a	****	n/a	*	n/a	n/a
%leaf	54	SS	0.0184	0.0123	0.0072	n/a	0.0084	n/a	0.0043	n/a	n/a
		P	****		***	n/a	***	n/a	ns	n/a	n/a
%stem	54	SS	0.1778	0.0371	0.1431	n/a	0.0120	n/a	0.0265	n/a	n/a
		P	****		****	n/a	*	n/a	**	n/a	n/a
%petiole	54	SS	0.0315	0.0119	0.0205	n/a	0.0023	n/a	0.0097	n/a	n/a
		P	****		****	n/a	ns	n/a	**	n/a	n/a
A (μmol m^{-2} s^{-1})	104	SS	1334	604	182	78	443	84	221	54	137
		P	****		***	**	****	*	**	ns	ns
A (kg m^{-2} s^{-1})	104	SS	1.857	0.654	0.411	0.049	0.647	0.028	0.343	0.085	0.105
		P	****		****	*	****	ns	***	*	ns
%N	95	SS	101.7	10.8	41.4	0.0	49.3	1.2	6.7	2.2	0.8
		P	****		****	ns	****	ns	***	**	ns
Δ (‰)	95	SS	69.68	10.35	58.43	2.10	2.17	2.27	3.65	0.09	0.98
		P	****		****	***	**	**	*	ns	ns

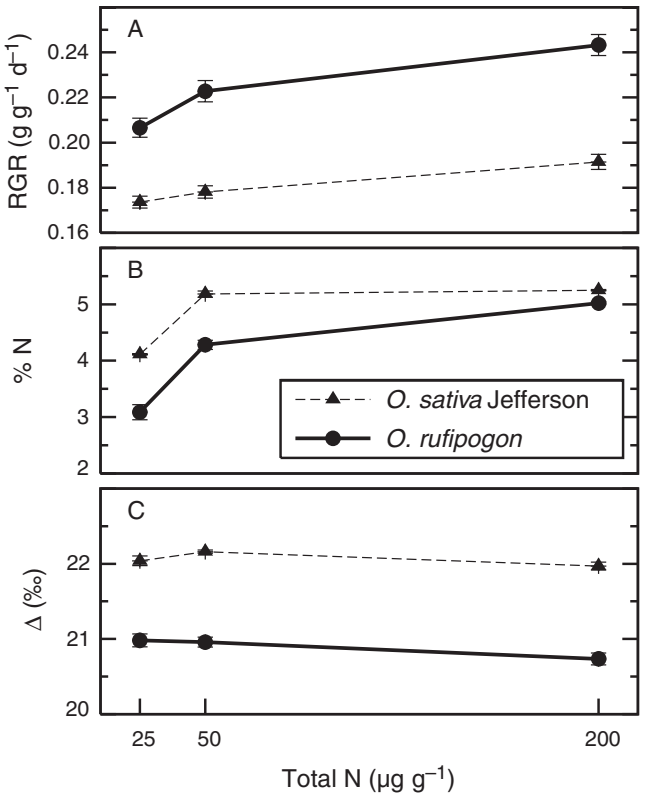

Fig. 3. Response of two rice genotypes to nitrogen concentrations. (*A*) The whole-plant relative growth rates, (*B*) %N measured for leaf-blade tissue only and (*C*) carbon isotope discrimination. Each data point represents the mean of six replicate plants. Error bars are ± one SE.

Nutrient status, and especially leaf nitrogen content, receives frequent attention in the context of WUE studies due to interest in whether variation in WUE, both among genotypes and / or environments, is related more to modulation of stomatal conductance or to changes in carboxylation capacity. High leaf nitrogen can theoretically promote simultaneous high photosynthetic rates and high WUE but this combination may be limited by whole-plant nitrogen budgets. A majority of crops studied have shown that genetic variation in Δ is related more strongly to variation in stomatal conductance than carboxylation capacity (Ehleringer *et al*. 1990; Turner 1993; White *et al*. 1994; Zacharisen *et al*. 1999) but several important exceptions have also been reported in both crop and wild species (Turner 1993; Rao *et al*. 1995; Virgona and Farquhar 1996; Udayakumar *et al*. 1998; Ares *et al*. 2000; Prasolova *et al*. 2003). Δ is sometimes positively correlated with both high stomatal conductance and high photosynthetic capacity through a suite of pleiotropic characters related to early vigor and maturation (McKay *et al*. 2003). Even in crops where overall correlations suggest that high yields are associated with high stomatal conductance and high Δ, individual genotypes may sometimes be found in which high yield is associated with low Δ (i.e. high water-use efficiency) under water-limiting conditions without sacrifice of maximal yield potential (Condon *et al*. 2002; Rebetzke *et al*. 2002).

Leaf nitrogen content is generally assumed to be strongly correlated with photosynthetic capacity (Reich *et al*. 1998, 1999), as was found here in tomato (Fig. 2), and so is frequently used as a proxy. In some studies, low nitrogen availability may result in reduced photosynthetic capacity without proportional stomatal closure and a concomitant reduction in Δ (Brück *et al.* 2001; DaMatta *et al*. 2002; DesRochers *et al*. 2003; Hamerlynck *et al*. 2004). However, we found that in both rice and tomato, large changes in leaf nitrogen were associated with only minor shifts in Δ even when leaf nitrogen was positively correlated with photosynthetic capacity. This indicates a strong co-regulation of stomatal and photosynthetic capacities in response to nutrient levels. The opposite trends of slightly increasing *v.* decreasing Δ in tomato (Fig. 2*L*, *M*) and rice (Fig. 3*C*), respectively, as %N increased may have been related to slight differences in the control of c_i or to differences in carbon metabolism and additional isotopic fractionation directly associated with nitrogen assimilation (Raven and Farquhar 1990; Guo *et al*. 2002). Nonetheless, Δ was largely stable with varying nitrogen availability, as has been seen in other studies (Livingston *et al*. 1999).

Soil media and flooding

Soil conditions were varied factorially in several experiments (e.g. Tables 2, 3), and generally showed no interaction with genotype in determining Δ. Both tomato and rice were tested across a comparison of mineral soil, used to facilitate root harvesting, and a more typical peat and vermiculite-based potting medium. *S. pennellii* was the only genotype, in either rice or tomato, that showed substantial sensitivity of Δ to soil type (e.g. Fig. 2*L*, *M*), including additional genotypes of both rice and tomato not detailed here (JP Comstock unpubl. data). The unique sensitivity of this genotype may be related to the unusual root characteristics of *S. pennellii*. This species, which originates in a fog-desert environment, has a very low overall allocation of biomass to the root system (Fig. 2*A*), and possesses extremely fine, hair-like roots.

Soil moisture conditions for cultivated rice range from flooded paddies to dry upland fields. This might influence comparisons of cultivars specialised to different practices. We therefore included flooded (anaerobic) and aerobic soil treatments for some experiments in rice (Table 3). Rice genotypes showed a small but significant main effect and no G × E in Δ for aerobic *v.* flooded conditions. Δ was slightly lower under flooded conditions, which is likely related either to stomatal closure or altered nitrogen uptake processes (Raven and Farquhar 1990).

Restrictive soil volume

In any scientific work done on potted material, the potential effects of root restriction should be taken into consideration. It is well established that root-to-shoot hormonal signalling is involved in stomatal responses to stress (Comstock 2002). This adds to concern that experimental results relating Δ to environmental conditions using potted material could be misleading in some circumstances. The effects of soil volume were therefore evaluated for contrasting pairs of tomato and rice genotypes (one cultivar and one genotype from a congeneric wild species in each case). Three soil volumes, 0.3, 0.8, and 2.5 L, were used in a factorial design with two nutrient levels indicated as 100 and 200 μg (N) L^{-1}.

Tomato

Both the cultivar E6203 and *S. habrochaites* showed substantial depressions of RGR with low soil volume, though E6203 was more sensitive (Table 5; Fig. 4*A*, *B*). Only E6203 showed significant enhancement of RGR at the higher nutrient level, consistent with results from the broader nutrient study reported above (Fig. 2*A*). Little of the overall growth suppression associated with restricted soil volume could be compensated by nutrient regime in either genotype (Fig. 4*A*, *B*). Instead, high nutrient availability caused elevated leaf nitrogen contents indicative of luxury consumption and storage (Fig. 4*I*, *J*). Allocation of biomass to roots was depressed at the high nutrient level (Fig. 4*E*, *F*). The percentage biomass allocated to the roots was similar in the two genotypes under most conditions but sharply elevated in *S. habrochaites* in the smallest soil volume at the lower nutrient level (Fig. 4*E*). Fixed genetic effects were again the most important determining factor for Δ. In contrast to the small nutrient and temperature effects discussed above, Δ showed substantial sensitivity to soil volume, particularly when soil volume was low (Fig. 4*M*, *N*). Values of Δ shifted, in some cases, more than 1‰, and significant genotype × soil volume interactions were observed (Table 5).

Rice

The high nutrient treatment enhanced growth slightly at all soil volumes in rice but did not compensate for the inhibition of limiting soil volumes (Table 6; Fig. 4*C*, *D*). Biomass allocation to roots was reduced at high nutrient levels but the response to pot size was variable. Root allocation increased in small pots in the low nutrient treatment (Fig. 4*G*) but decreased in small pots in the high nutrient treatment (Fig. 4*H*). Leaf nitrogen content was positively correlated with RGR in rice; it was lowest for small soil volumes and low nutrient levels. In rice, Δ was again primarily determined by genotype with a slight depression at high nutrients (Fig. 4*O*, *P*) consistent with the trend reported above (Fig. 3*C*).

In both crops, constriction of root expansion in limiting soil volumes caused a growth restriction despite the maintenance of well watered conditions, compensating nutrient regimes, and adequate spacing to avoid crowding and light competition (Fig. 4*A*, *B*, *C*, *D*). This suggests that the restriction was due to hormonal signals from the roots leading to down-regulation of growth rates. Substantial effects on Δ were seen only at very small soil volumes at 3–4 weeks of age. Tomato was more sensitive than rice to small soil volumes, showing greater RGR inhibition and a more dramatic Δ response (Fig. 4*M*, *N*, *O*, *P*).

Plant age and soil volume

In other experiments reported here, plants were sampled at only a single age, always between 3 and 4 weeks from germination. The generality of these age-specific results could be influenced both by inherent developmental patterns and changing plant size relative to soil volume. To address these issues, a second experiment involving variable soil volume was evaluated in rice, this time contrasting two cultivated *O. sativa* genotypes, Nipponbare and Kasalath. In this experiment, plants were grown with a wider range of soil volumes spanning 0.3–35.0 L and were sampled repeatedly at ages 2–5 weeks (Fig. 5).

Table 5. The effects on tomato of three soil-volumes and two nutrient regimes in factorial design

The stability of genetic ranking of two tomato genotypes, *S. lycopersicum* E6203 and *S. habrochaites*, were evaluated for growth rate, allocation, and Δ (Fig. 4*A*, *B*, *E*, *F*, *I*, *J*, *M*, *N*). Sample sizes, variable, column, and row definitions are similar to Table 2

Parameter	df		Model	Error	Genotype df = 1	Volume df = 2	Nutrient df = 1	G × V df = 2	G × N df = 1	V × N df = 2	G × V × N df = 2
RGR ($g\,g^{-1}\,day^{-1}$)	68	SS	0.0881	0.0107	0.0445	0.0398	0.0015	0.0018	0.0009	0.0006	0.0003
		P	****		****	****	**	*	*	ns	ns
%root	68	SS	0.0918	0.0542	0.0064	0.0011	0.0659	0.0042	0.0040	0.0035	0.0049
		P	****		*	ns	****	ns	*	ns	ns
%N	35	SS	50.32	1.80	3.66	3.32	40.52	0.29	1.25	0.53	0.75
		P	****		****	****	****	ns	***	*	*
Δ (‰)	35	SS	38.80	3.14	24.87	4.22	4.93	1.50	0.33	2.32	0.64
		P	****		****	****	****	**	ns	**	ns

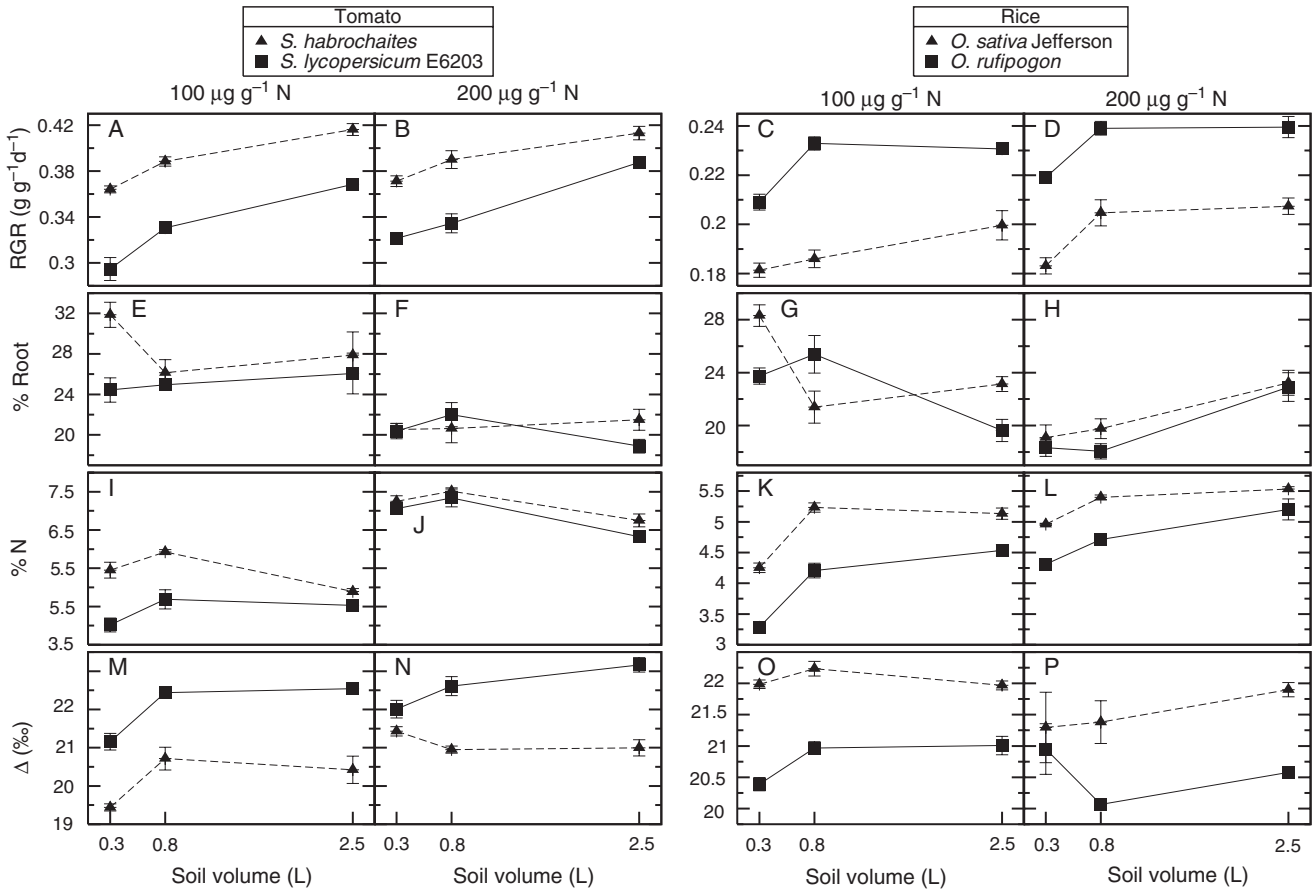

Fig. 4. Effects of soil volume and fertiliser concentration on tomato and rice genotypes. This shows responses of both tomato (panels *A*, *B*, *E*, *F*, *I*, *J*, *M*, *N*) and rice (panels *C*, *D*, *G*, *H*, *K*, *L*, *O*, *P*) to a factorial treatment of soil volume and fertiliser level. Different fertiliser levels are shown in the contiguous columns of panels, and soil volume is the *x*-axis throughout. Plot symbols refer to contrasting genotypes in each crop. Panels *A*, *B*, *C*, *D*: whole-plant relative growth rate; *E*, *F*, *G*, *H*: %root; *I*, *J*, *K*, *L*: %N; *M*, *N*, *O*, *P*: carbon isotope discrimination. Each data point represents the mean of six replicate plants. Error bars are ± one SE.

Table 6. The effects on rice of three soil-volumes and two nutrient regimes in factorial design

The stability of genetic rankings of two rice genotypes was evaluated for growth rate, allocation, and Δ. Rice varieties include *O. sativa* Jefferson and *O. rufipogon* (Fig. 4*C*, *D*, *G*, *H*, *K*, *L*, *O*, *P*). Sample sizes, variable, column, and row definitions are similar to Table 2

Parameter	df		Model	Error	Genotype df = 1	Volume df = 2	Nutrient df = 1	G × V df = 2	G × N df = 1	V × N df = 2	G × V × N df = 2
RGR (g g^{-1} day^{-1})	71	SS	0.0300	0.0047	0.0217	0.0061	0.0014	0.0003	0.0000	0.0001	0.0003
		P	****		****	****	****	ns	ns	ns	ns
%root	71	SS	0.0637	0.0295	0.0024	0.0021	0.0204	0.0049	0.0001	0.0254	0.0085
		P	****		*	ns	****	*	ns	****	***
%N	35	SS	13.74	0.49	4.53	5.30	2.99	0.28	0.21	0.43	0.00
		P	****		****	****	****	**	**	***	ns
Δ (‰)	35	SS	15.84	4.10	11.58	0.33	1.41	0.15	0.18	1.09	1.09
		P	****		****	ns	*	ns	ns	ns	ns

Within a cultivar, values for Δ were most similar across soil volumes at the earliest ages, but pronounced soil volume effects developed in both cultivars as plants aged (Fig. 5*A*, *B*). This was most notable in Kasalath, where the values of Δ for plants in the two smallest volumes had 1‰ higher discrimination at 5 weeks (Fig. 5*B*). In Nipponbare, differences in Δ due to soil volume were also more pronounced with age but never so large (Fig. 5*A*). While older Kasalath plants had higher Δ in small soil volumes, the opposite was true in Nipponbare. Consequently, at 2 and

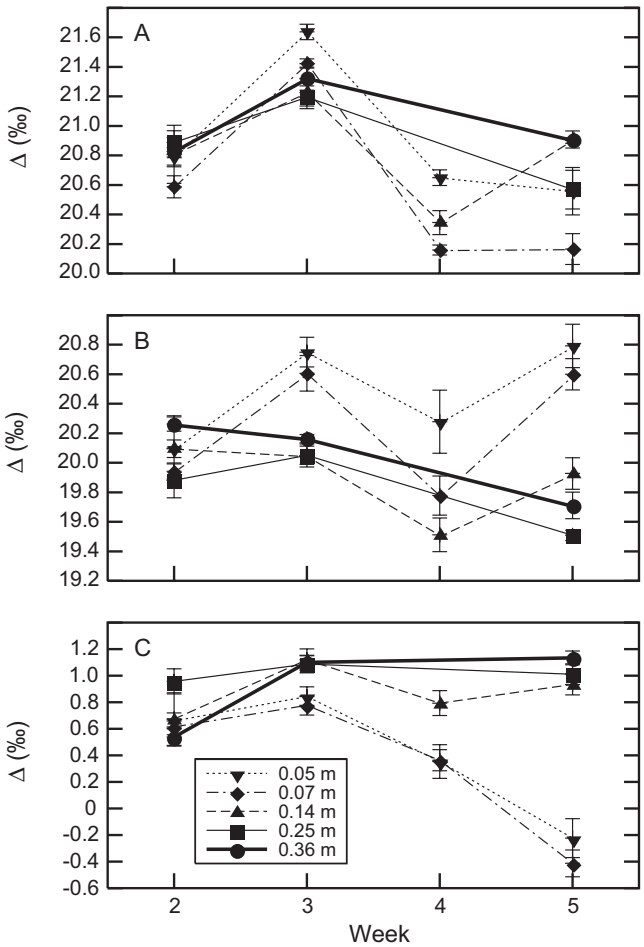

Fig. 5. Stability of Δ with age in young vegetative rice in a wide range of pots and associated soil volumes. (*A*) *O. sativa* Nipponbare. (*B*) *O. sativa* Kasalath. (*C*) The difference in Δ between the two cultivars (Nipponbare – Kasalath) as calculated for each pot size on each date. Plot symbols indicate pots of contrasting diameter: 0.05, 0.07, 0.14, 0.25, and 0.36 m, which contained soil volumes of 0.33, 0.30, 2.5, 13.0, and 35.0 L, respectively. Data points represent the means of six replicate plants, and error bars are ± one SE.

3 weeks, genetic differences in Δ were almost independent of soil volume (Fig. 5*C*). With increasing age, the two genotypes continued to have the same stable relationship for Δ in the three largest soil volumes but actually reversed rank for Δ in the smallest soil volumes. Total soil volume seemed to be of greater importance than the dimension ratio, at least under this relatively extreme condition (see pot-size description in Materials and methods). Significant effects on plant growth rates, as indicated by plant height and tiller number, were discernable earlier than effects on Δ and were associated with the two smallest soil volumes even at 2 weeks of age (data not shown).

The severe root restriction generating strong G × E in Δ would be unlikely under most crop field conditions but has considerable practical importance when measuring Δ in glasshouse or growth chamber conditions where space is limited. Small soil volumes gave comparable results to larger pots only at very young ages, and led to substantial changes in genetic rankings with age. Plants in a soil volume of 2.5 L had growth rates and Δ indistinguishable from those of plants grown in much larger soil volumes, at least up to 5 weeks of age. This was the standard soil volume used in most experiments reported here, and the plants were screened at an age of 3–4 weeks.

Irradiance levels

We investigated the response to varying growth irradiance (light) levels from ~400 to 1400 μmol (PAR) m^{-2} s^{-1} for both rice and tomato. The lowest light levels averaged one-third or less of photosynthetic light saturation while the highest levels were fully saturating throughout the photoperiod. Growth light level resulted in greater variation in Δ than any other environmental parameter tested, showing changes of over 2‰ across the tested range (Fig. 6*A*, *B*). While genotype × irradiance interactions were not significant in the experiments shown here, we have observed genotype-specific sensitivity to light levels for these genotypes in other experiments (JP Comstock, unpubl. data). Owing to the large magnitude of the irradiance effects on Δ, consistency of known irradiance levels is perhaps the single most important environmental control for any genetic comparisons of values in this trait.

Irradiance level and shading have been observed, in previous studies, to have effects on Δ similar in magnitude and direction to those observed here (Ehleringer *et al*. 1986; Hanba *et al*. 1997; Carelli *et al*. 1999). Growth irradiance levels can alter several different physiologically important traits, including electron-transport rate, direct stomatal responses to light, and responses associated with leaf temperature. In our experiments with both crops, air temperature was constant but leaf temperatures may have varied as much as 2°C, having a substantial effect on the leaf-to-air pressure gradient, *D* (Fig. 6). It is likely that the responses to irradiance treatments we observed were a mixture of direct responses to light and additional responses to *D*, both acting to lower Δ at high irradiance. This complex response to irradiance level, and the magnitude of its effect on Δ, suggest that irradiance should be kept as uniform as possible when measuring Δ. Since there is some evidence for genotype by environment interactions for this trait, although it was not strong in the studies reported here, it appears that careful control of irradiance levels, and use of irradiance levels appropriate to field growing conditions, would be desirable for accurate characterisation of genetic variation in Δ.

Conclusions

For most environmental conditions tested, genetic rankings for Δ showed remarkably little variation and, regardless of

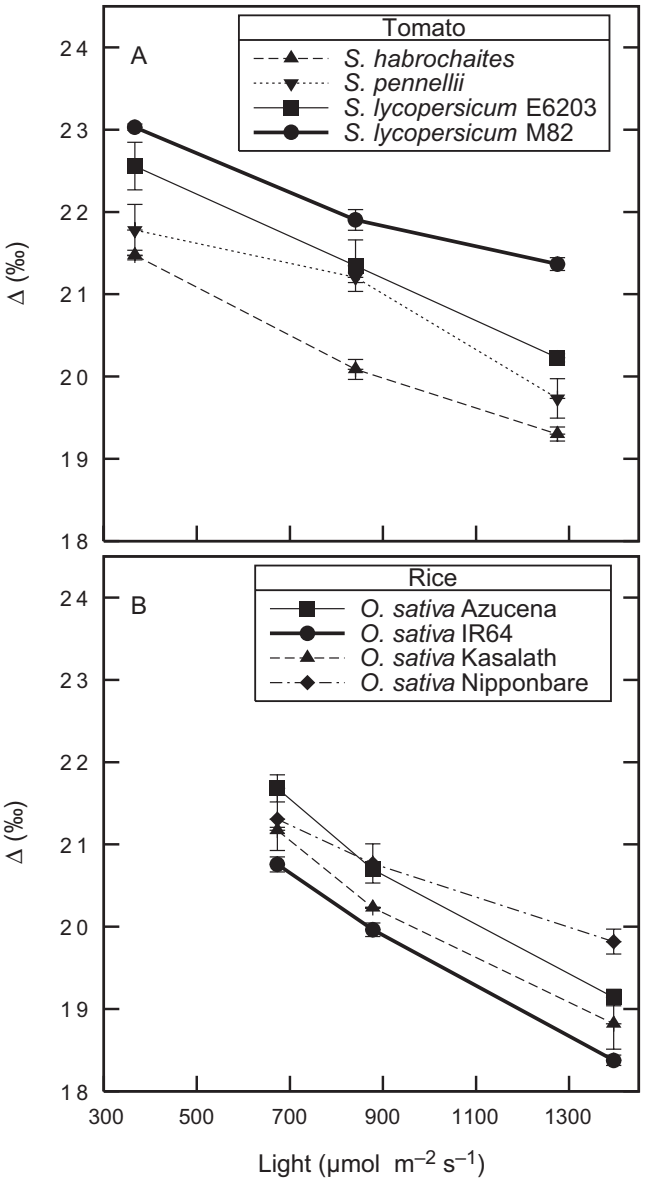

Fig. 6. Effects of growth irradiance on Δ in selected tomato (*A*) and rice (*B*) genotypes. All plants were grown together in a glasshouse but received varying degrees of supplemental HID lamp output. Light levels given are mean photoperiod irradiances of total PAR. Data points represent the means of six replicate plants, and error bars are ± one SE.

significance levels, sums of squares for G × E interaction terms were usually very small relative to those for genotypic and environmental main effects. The genotypes in these studies were specifically chosen because they were known to provide contrasts in Δ under at least some previously tested conditions (Martin *et al*. 1989; Dingkuhn *et al*. 1991*b*; JP Comstock unpubl. preliminary surveys) and because of their potential relevance to future studies as parents of available permanent mapping populations (Eshed and Zamir 1994; Huang *et al*. 1997; Lin *et al*. 1998; Ishimaru *et al*. 2001; Monforte and Tanksley 2000). While similar studies of genotypes with less underlying genetic difference in Δ setpoints would be likely to show a greater sensitivity of ranking to environmental factors, these studies have demonstrated that genetic differences for Δ in rice and tomato may be quite robust over a wide range of conditions.

The conservative genetic rankings of Δ are highly consistent with the view that this parameter is indicative of a specifically controlled set-point between stomatal conductance and the rate of photosynthetic carbon metabolism. Several such examples are presented above, with the most dramatic being the tomato responses to nutrient levels (Fig. 2; Table 4). The two domestic cultivars showed 3-fold changes in photosynthetic rate per unit mass coupled with large changes in SLA, resulting in almost 2-fold changes in the photosynthetic rate per unit area. Nonetheless, the strong main effects and G × E in these mechanistically linked traits did not alter the final ratio of leaf diffusive exchange relative to carboxylation capacity as indicated by Δ. This implies a stomatal feedback mechanism that is very finely tuned to compensate for these altered balances and is regulated to different setpoints in the contrasting genotypes.

Such feedback is likely to be mediated by several mechanisms operating simultaneously during growth. At the shortest timescale is direct sensing of leaf internal CO_2 concentration (Mott 1988, 1990; Assmann and Palade 1993). Feedback cues are also derived from metabolite pools downstream of the initial carbon fixation steps in the chloroplasts (Paul and Foyer 2001), and may affect developmental processes in the leaf (Sage 2000; Brownlee 2001) as well as short-term stomatal regulation. Whatever the mechanism and regardless of whether the feedback response is short- or long-term, regulatory feedback linked to c_i will result in the coordination of photosynthetic capacity and stomatal behaviour with a genetically determined set-point indicated by Δ.

In contrast to the low and usually non-significant levels of G × E observed for Δ in these experiments, environmental variables commonly did have main effects of various magnitudes. Main environmental effects were small for nutrient level in both rice and tomato, soil flooding in rice, and temperature treatments in tomato. Larger temperature effects were seen in rice, but primarily at a substantially sub-optimal growth temperature. The narrow range of Δ with respect to environmental variation in these cases is also consistent with the hypothesis that Δ reflects a broad set-point in metabolism.

The largest departure from this pattern is seen in the irradiance studies where main treatment effects spanned nearly 2‰. Based on the dynamics of diffusion gradients, c_i must inevitably pass from values higher than c_a during

dark respiration and irradiance levels below the carbon assimilation compensation point (i.e. $A \leq 0$) to values lower than c_a as A becomes positive. In leaf gas-exchange studies, c_i usually stabilises at higher light levels, but may show progressive changes when light is less than half-saturating for carbon assimilation (Huxman and Monson 2003). Light is also highly studied in guard cell signal transduction as one of the primary cues for stomatal opening and short-term aperture regulation (Assmann and Palade 1993; Dietrich *et al*. 2001). Set-point behaviour reflected by Δ is therefore consistent with feedback regulation related to c_i as discussed above. It does not, however, imply full homeostasis in c_i when interacting with other stomatal regulatory mechanisms (Huxman and Monson 2003). Finally, large changes in A at low *v.* high light levels can alter the relationships between c_a, c_i, and the [CO_2] in the chloroplasts (c_c), and may affect Δ in ways more complex than captured in Eqn 3 (Evans and von Caemmerer 1996).

Despite the lack of strong $G \times E$ responses in Δ, the numerous fixed responses of Δ to environmental conditions indicate a practical need for tight environmental control during any genetic screening activities. In terms of the magnitudes of fixed shifts in Δ associated with different environmental conditions, irradiance level > temperature > nutrient level > soil media conditions. All of these variables need to be monitored and held constant. Irradiance deserves special attention because of the magnitude of potential effects on Δ and because it is one of the most difficult variables to standardise in a glasshouse context.

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