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Use of introgression lines to determine the ecophysiological basis for changes in water use efficiency and yield in California processing tomatoes

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Abstract. Field and greenhouse studies examined the effects of growth habit and chloroplast presence in leaf veins for their role in increasing agronomic water use efficiency and yields of California modern processing tomato (*Solanum lycopersicum* L.) cultivars. Five introgression lines (ILs), made with *Solanum pennellii* Cor. in the genetic background of cultivar M82, differ in genes that map to a region on Chromosome 5, including the *SP5G* gene (determinate *vs.* semideterminate (Det *vs.* SemiDet)) and the *obv* gene (presence (obscure) *vs.* absence (clear) of leaf vein chloroplasts (Obs *vs.* Clr)). The five ILs and M82 represented three of the four gene combinations (Det–Clr was unavailable). Det–Obs ILs had less leaf, stem and total aboveground biomass with earlier fruit set and ripening than SemiDet–Clr ILs. By harvest, total fruit biomass was not different among ILs. Photosynthetic rates and stomatal conductance were 4–7% and 13–26% higher, respectively, in Det–Obs ILs than SemiDet–Clr ILs. SemiDet–Obs ILs were intermediate for growth and gas exchange variables. The Det–Obs ILs had lower leaf N concentration and similar chlorophyll content per leaf area (but slightly higher per leaf mass) than SemiDet–Clr ILs. The Obs trait was associated with gains in leaf gas exchange-related traits. This study suggests that a more compact growth habit, less leaf biomass and higher C assimilation capacity per leaf area were relevant traits for the increased yields in cultivars with determinate growth. Developing new introgression libraries would contribute to understanding the multiple trait effects of desirable phenotypes.

Additional keywords: chloroplasts, determinate growth, leaf veins, *Solanum lycopersicum*, *Solanum pennellii*.

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Introduction

Crop breeding has played an important role in increasing yields, but the mechanisms by which particular morphological, anatomical and physiological traits contribute to these higher achieved yields are not well understood for many crops. Large yield increases are often the result of an interaction between crop improvement and new agronomic practices, such as the introduction of the dwarfing genes and higher nitrogen inputs in wheat (*Triticum aestivum* L.), or more determinate growth and mechanised harvest in processing tomatoes (*Solanum lycopersicum* L.) (Stevens and Rick 1986; Evans and Fischer 1999). More emphasis is needed on how specific traits are associated with yield gains in order to overcome future potential environmental constraints (e.g. diminishing water supply) (Passioura 2002; Fischer 2007). The use of genomic libraries comprised of introgressed chromosome segments from a crop wild relative has become a powerful tool for understanding how specific chromosome regions affect traits of interest (Gur and Zamir 2004). In tomatoes, a library of 76 introgression lines (ILs), each containing a single chromosome segment from the wild species, *Solanum pennellii* Cor., has been developed (Eshed and Zamir 1994). The ILs are nearly isogenic in cultivar 'M82', a determinate processing tomato bred for mechanized harvest in the 1970s. Using this genetic material, leaf traits that affect photosynthetic rates and plant growth habit can be further explored as sources of genetic variation to improve crop yields in the future (Poorter and Evans 1998; Evans and Poorter 2001; Tanaka *et al*. 2008).

The determinate growth habit was introduced into California processing tomato cultivars starting in the 1930s (Porter and MacGillivray 1937) and became widely adopted following the shift to mechanical harvest in the 1960s. Average productivity (tonnes ha^{-1}) has more than doubled since then (United States Department of Agriculture 2009). Determinate cultivars have a compact canopy and a more defined vegetative and reproductive stage that allows for more synchronous fruit production and

thus higher yields from mechanical (i.e. a destructive, one-time) harvest (Stevens and Rick 1986). Along with these plant architectural and phenological changes, evapotranspiration rates have remained constant but agronomic water use efficiency (yield per applied water; WUE_a) has increased by more than 50% in the last four decades (Hanson and May 2006). This gain in WUE_a may have resulted from agricultural practices (e.g. planting density and disease management), in combination with greater plant physiological potential (e.g. hybrid cultivars) (Grandillo *et al*. 1999) to increase yields. Little is known, however, about the physiological basis for WUE_a and yield increases after the introduction of a determinate growth habit in processing tomatoes.

In tomato, the switch between indeterminate and determinate growth is controlled by the *self-pruning* (*SP*) gene located on Chromosome 6; but five other members of the *SP* gene family are present in the genome and may also affect the expression of determinacy (Carmel-Goren *et al*. 2003). Many of the currently used cultivars vary in the severity of their determinate growth habit and have larger canopy size than very determinate cultivars such as M82, which was released in the 1970s (Gur *et al*. 2010). The *SP5G* gene is located in bin 5G of Chromosome 5 (Jones *et al*. 2007). The IL 5–4 line carries the *SP5G* allele of *S. pennellii* and has a less determinate growth habit (i.e. semideterminate as compared with M82) with higher plant weight and height and more fruits that were green (Eshed and Zamir 1995; Jones *et al*. 2007). Very close to the *SP5G* gene and in the same 5G bin is the *obscuravenosa* (*obv*) gene (Jones *et al*. 2007). The wild-type allele of *obv* (i.e. obv^+) confers veins that transmit light and appear lighter coloured than the leaf lamina due to the absence of chloroplasts in the veinal tissue. The mutant allele, *obv*, increases the density of chloroplasts in leaf veins, resulting in 'obscure' veins (i.e. same colour as the leaf lamina). The veins of *obv* are also less recessed in the leaf, resulting in a flatter leaf surface. (Hereafter, obscure (Obs) and clear (Clr) veins will be used to refer to *obv* and +, respectively, and determinate (Det) and semideterminate (SemiDet) will be used to refer to *sp5g* and +, where + indicates the the wild-type alleles from *S. pennellii*). The *obv* mutation became more widespread in processing tomato cultivars with the introduction of determinate growth in the 1930s (Jones *et al*. 2007). Even after the introduction of hybrids in the 1990s, the *obv* trait is still present in ~50% of cultivars, indicating that both parents of these hybrids are homozygous *obv/obv*. It is not clear why the obscure vein trait persists in processing cultivars. One possibility is that the *obv* gene has been selected for because it is linked to a beneficial allele at the *SP5G* locus. Alternatively, *obv* might confer a direct physiological advantage, such as for carbon assimilation.

Leaf anatomical and morphological traits can affect chlorophyll content, which is correlated with higher photosynthetic rates (Emerson 1929; Fleischer 1935; Watanabe *et al*. 1994; Murchie *et al*. 2009). In many crops, an increase in photosynthetic rates (P_n) is associated with an increase in stomatal conductance (g_s) and thus greater CO_2 diffusion to the sites of carboxylation, C assimilation and growth (Morgan *et al*. 1990; Fischer *et al*. 1998; Terashima *et al*. 2005). In irrigated environments, a tradeoff associated with higher P_n is a reduction in the intrinsic WUE ($P_n\ g_s^{-1}$; WUE_i), which generally favours higher yields (Condon *et al*. 2004). WUE_i can be determined from instantaneous leaf gas exchange measurements, and plant ^{13}C discrimination ($\Delta^{13}C$) values can be used as a surrogate for cumulative WUE_i in C_3 plants (Farquhar *et al*. 1982; Farquhar and Richards 1984). In tomatoes, $\Delta^{13}C$ has been shown to be a reliable indirect measure of WUE_i under different environmental conditions (Martin *et al*. 1999; Comstock *et al*. 2005; Xu *et al*. 2008). Thus isogenic lines that differ in the occurrence of leaf vein chloroplasts may differ in both leaf gas exchange and $\Delta^{13}C$.

Changes in growth habit within a species may also affect C assimilation potential and capacity (e.g. in soybean (*Glycine max* (L.) Merr.) and wheat (LeCain *et al*. 1989; Morgan *et al*. 1990; Tanaka *et al*. 2008). For instance, an increase in the density of photosynthetic machinery in a semidwarf wheat genotype increased CO_2 assimilation rates compared with its 'Tall' near-isogenic line (LeCain *et al*. 1989). Greater specific leaf area (SLA) correlates with higher photosynthetic rates per unit leaf mass in many species including *S. lycopersicum* (Poorter and Evans 1998; Comstock *et al*. 2005). Nitrogen content and partitioning within a leaf, and their effects on P_n, can be influenced by plant growth habit, growing conditions or SLA (Fischer *et al*. 1998; Poorter and Evans 1998; Evans and Poorter 2001).

We hypothesise that the gains in yield of modern tomato cultivars are due to a suite of traits that include a change in biomass allocation, determinate growth and an increase in C assimilation capacity related to leaf traits. A group of ILs differing in growth habit as influenced by the *SP5G* gene (Det *vs.* SemiDet) and the presence of chloroplast in leaf veins (*obv* gene; Obs *vs.* Clr) were compared in field and greenhouse studies. Three out of the four possible gene combinations (i.e. growth habit × chloroplast presence in leaf veins) were available for these studies; ILs with determinate growth habit and without leaf vein chloroplasts (Det–Clr) were not available. The specific objectives were to: (1) assess the IL combinations of the *SP5G* and *obv* genes on growth habit, biomass allocation and leaf gas exchange measurements; (2) explore other differences in leaf morphological and physiological traits amongst the ILs; and (3) evaluate if modern tomato cultivars with or without chloroplasts in the leaf veins differed in leaf gas exchange traits, growth and biomass allocation. Experiments were conducted at very low planting densities to favour full plant development and to avoid resource limitation from plant-to-plant competition. Different planting times in the two field experiments were chosen to evaluate how plant performance was affected by mild versus warmer temperatures (spring *vs.* summer planting dates).

Materials and methods

Plant material

Introgression lines

A total of six tomato lines were used in the field and greenhouse studies: five ILs and cultivar M82 (Table 1). These ILs have defined chromosome segments from the *Solanum pennellii* Cor. genome in the genetic background of a cultivated processing tomato, *Solanum lycopersicum* L. cv. M82. Detailed descriptions of the ILs and of the IL 5–4 region

Table 1. List of tomato genotypes used in these studies

Cultivar M82 (*Solanum lycopersicum*) and introgression lines containing *Solanum pennellii* chromosome segments in an M82 background are classified for specific traits determined by the *SP5G* gene for determinate or semideterminate growth habit, and the *obv* gene for the presence (Obscure) or absence (Clear) of leaf vein chloroplasts. Hybrid cultivars show their trait for the presence or absence of leaf vein chloroplasts. Days to maturity and the source of the seeds are included. LA# is the accession number from the C.M. Rick Tomato Genetics Resource Center at UC Davis

Plant material	Growth habit[A]	Leaf vein	Days to maturity	Source
Introgression line				
M82[B]	Determinate	Obscure	105	LA3475
IL 5–4–4	Determinate	Obscure		LA4436
IL 5–4–1	Semideterminate	Obscure		LA4434
IL 5–4	Semideterminate	Clear		LA4057
IL 5–4–2	Semideterminate	Clear		LA4435
IL 5–4–5–44	Semideterminate	Clear		LA4429
Hybrid cultivars				
CXD-206		Obscure	114	Campbells (Davis, CA, USA)
AB2		Obscure	120	DeRuiter (St Louis, MO, USA)
HP-303		Obscure	128	Seminis (St Louis, MO, USA)
Sun-6397		Obscure	116	Nunhems (Parma, ID, USA)
Sun-6368		Obscure	125	Nunhems
BOS-67374		Obscure	125	Orsetti (Hollister, CA, USA)
APT-410		Obscure	114	Seminis
PX-002		Obscure	125	Seminis
CXD-255		Clear	125	Campbells
HM-9905		Clear	125	Harris Moran (Davis, CA, USA)
H-9780		Clear	138	Heinz (Pittsburgh, PA, USA)
H-2601		Clear	122	Heinz
H-3402		Clear	120	Heinz
HP-108		Clear	128	Seminis
HP-849		Clear	130	Seminis
SVR-1245		Clear	118	Monsanto (St Louis, MO, USA)

[A]Growth habit is not defined for hybrids.
[B]Cultivar M82 is the genetic background for the introgression lines.

are provided elsewhere (Eshed and Zamir 1994; Jones *et al.* 2007; Xu *et al.* 2008). For our studies, five unique ILs were chosen based on determinacy and the presence of chloroplasts in leaf veins, which are traits associated with the *SP5G* and the *obv* genes, respectively. The ILs represent all but one of the possible trait combinations (Table 1). Seeds of the ILs and M82 were obtained from the C. M. Rick Tomato Genetics Resource Center at University of California (UC) Davis (LA numbered accessions). All seeds were surface sterilised and germinated in trays, and seedlings were maintained under a day : night length of 16 : 8 h in UC Davis greenhouses. Seedlings were transplanted to either pots or in the field when they had at least three true leaves (i.e. 6-week-old plants).

Hybrid cultivars

An additional field experiment evaluated the effects of the presence or absence of chloroplasts in leaf veins on cultivars currently used by the California processing tomato industry. Eight cultivars for each trait (i.e. presence *vs.* absence of chloroplasts in leaf veins; a total of 16 cultivars) were selected based on Jones *et al.* (2007) and collaborators from the tomato industry. All cultivars were hybrids. From here onwards, this experiment is referred as the 'Hybrid cultivars' experiment.

Field studies: Experimental design

Introgression lines

Two field studies using typical management practices for processing tomatoes in California were conducted during the summers of 2009 and 2012 (Year 1 and Year 2, respectively) at the UC Davis Plant Sciences Research Station in Davis, California. In Year 1, the soil was mapped as a Reiff very fine sandy loam, a coarse-loamy, mixed, nonacid, thermic Mollic Xerofluvent (UC Davis 2013). In Year 2, the soil was mapped as a Yolo silt loam, a fine-silty, mixed, nonacid, thermic Typic Xerorthents. In Year 1's experiment, from 13 July to 25 September 2009, the average solar radiation was 293 W m^{-2}, the minimum and maximum average temperatures were 13.1°C and 34.3°C, respectively, with a minimum of 8.3°C and a maximum of 40.6°C, and no rainfall (California Department of Water Resources 2012). In Year 2's experiment, from May 7 to August 30, the same variables were 334 W m^{-2}, 11.8°C and 31.1°C, respectively, with extremes of 7.2°C and 39.4°C, and no rainfall (California Department of Water Resources 2012).

In both years, the fields were tilled and beds were prepared (1.52 m from furrow to furrow) for transplanting in spring. In each year, six replicate plots of the ILs were planted. In Year 1, a total of nine beds were divided in three groups of one IL bed and one buffer bed on each side. Each IL bed had a total of 12 6-m

plots; two plots for each IL per bed (six plots total per IL in the whole experiment). In Year 2, six IL beds were planted with no buffer bed between IL beds and each bed had a total of six 4.2-m plots, one per IL (six plots total per IL in the whole experiment). Tomato seedlings were hand-transplanted in the centre of the bed at a spacing of 1.2 m between plants in Year 1 (a total of five plants per plot) and 0.6 m between plants in Year 2 (a total of seven plants per plot). Plant density was about four and two times less for Year 1 and 2, respectively, than is commonly used in commercial production to minimise competition for light, water and nutrients. In Year 1, plants were sprinkler-irrigated several times after transplanting to assure good establishment; the mean maximum temperature during the first 2 weeks of plant establishment was 36.4°C (summer planting date). Four furrow irrigations followed until harvest on 25 September (74 days after transplanting, DAP) at intervals of ~12 days. In Year 2, plants were sprinkler-irrigated only once. Furrow irrigation started at 3 DAP and a total of 10 irrigations were applied until harvest on 30 August (115 DAP) at intervals of ~12 days; the mean maximum temperature during the first 2 weeks of plant establishment was 29.8°C (spring planting date).

Hybrid cultivars

In Year 2, the comparison of 16 cultivars used an experimental design similar to Year 2's IL experiment, with the same planting time, soil type, management and irrigation. However, two adjacent beds constituted one replicate (eight plots per bed and 16 plots total per replicate). Four replicate plots for each cultivar were planted in eight beds (64 plots in total).

For all the IL and hybrid cultivar field experiments, all physiological measurements were taken within a 34-day period of maximum plant growth (i.e. at the transition between vegetative growth and the onset of flowering and fruit set). In Year 1, plants were harvested at 74 DAP (premature harvest) because the high humidity and cool temperatures of late summer are conducive to disease, and can reduce plant growth and fruit development. In Year 2, IL plots were harvested at 115 DAP and hybrid cultivars at 120 DAP (normal harvest), which is common for processing tomatoes in California.

Field studies: Shoot growth, development and biomass

Introgression lines

In both years, canopy cover was measured using an agricultural digital camera (ADC, Tetracam Inc., Chatsworth, CA, USA). In Year 1, 1.0-m^2 images of a single plant were taken at 46 DAP and 65 DAP, and processed with Briv32 ver. 1.27 software (Tetracam Inc.) to obtain the fraction of the soil surface covered by the canopy (% cover m^{-2}). In Year 2, 1.8-m^2 images of two adjacent plants were taken at 11 DAP, 49 DAP, 77 DAP, 99 DAP and 108 DAP with an ADC Lite camera (Tetracam Inc.) and processed with PixelWrench II ver. 1.0.7.5 software (Tetracam Inc.). In Year 1, canopy light interception was measured at 44 DAP and 64 DAP using a portable-tube solarimeter with sensors for PAR (AccuPAR-80 ver. 4.5, Decagon Devices Inc., Pullman, WA, USA). At 44 DAP, each plant measurement was the composite of seven PAR reading intervals to encompass an entire plant's canopy (12.5 cm between intervals and a total area of 0.7 m^2). At 64 DAP, measurements for a full canopy was determined in a 2.3-m^2 area with PAR reading intervals at 15 cm along the bed. At each interval, a PAR reading was taken above and below the canopy, covering the entire width of the bed top to encompass the full canopy. Data are expressed as the percent of light intercepted per square meter (% PAR intercepted m^{-2} $plant^{-1}$). Since these results showed a high correlation with canopy cover (see below), canopy light interception was not measured in Year 2.

At the end of the experiment in Year 1 (74 DAP), the height of the middle plant in every plot was measured. The plant was clipped at the base of the stem; sorted into leaves, stems and fruits; and oven-dried at 60°C. Samples were analysed for total C and N content with an ECS 4010 CHNSO analyser (Costech Analytical Technologies Inc., Valencia, CA, USA). At the end of the experiment in Year 2 (115 DAP), two plants per plot were harvested; sorted into shoots, harvestable fruit and green or unripe fruit; dried and analysed for C and N content as in Year 1. Harvest index (HI) was calculated as the percent of total dry fruit biomass (harvestable + green or unripe fruit) from the total dry aboveground biomass (shoot + fruit).

Hybrid cultivars

Canopy cover measurements were taken at 49 DAP, 77 DAP and 99 DAP, and biomass harvest was conducted over 2 days, 120 and 121 DAP. Procedures were exactly as described above for Year 2.

Field studies: Leaf gas exchange and $\Delta^{13}C$

Introgression lines

In both years, leaf gas exchange measurements were taken on a mature, fully expanded leaflet from the top of the canopy, measured with a field portable open flow infrared gas analyser (model 6400, LI-COR Inc., Lincoln, NE, USA). Measurements were taken between 1015 hours and 1315 hours with a 6-cm^2 chamber, with the CO_2 reference set at 400 μmol m^{-2} s^{-1} and with saturating light using a light-emitting diode source (PAR in 2000 μmol m^{-2} s^{-1}). The first measurements were done at the onset of flowering and when canopy cover was still similar among ILs (see results below). In both years, measurements were taken in three 3-day runs. In Year 1, 9 total days with 24 plants per day were measured (i.e. six ILs each with four plots). In Year 2, 12 total days with 30 plants per day were measured (i.e. six ILs each with five plots).

The leaflets that had been measured for gas exchange were analysed for $\Delta^{13}C$ in both years. In Year 2, the leaflets were used for SLA and N analyses (see below). Samples were analysed for $\delta^{13}C$ and total N on a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd, Cheshire, UK) at the UC Davis Stable Isotope Facility (http://stableisotopefacility.ucdavis.edu/). $\Delta^{13}C$ was calculated from the $\delta^{13}C$ values using an air $\delta^{13}C$ value of –8‰ as described by Comstock *et al.* (2005).

Hybrid cultivars

Leaf gas exchange measurements were taken in four 2-day runs with 8 total days of measurements. Each day, 32 plants were measured (two replicate plots per cultivar) such that 64 plots

were measured in each 2-day run. $\Delta^{13}C$ was measured on leaflets saved from gas exchange measurements as described above.

Field studies: Leaflet morphology and chlorophyll content

Introgression lines

Similar and adjacent leaflets to those used for gas exchange measurement were sampled for stomatal density, SLA and chlorophyll content.

Stomatal density was determined by sampling whole leaflets, and imprints prepared for the abaxial and adaxial sides (Gailing *et al*. 2008). A total of 72 samples were collected in year 1 (six ILs × six plots × two plants per plot), and 36 samples in Year 2 (six ILs × six plots × one plant per plot). The number of stomata was counted from microphotographs using an Eclipse E600 microscope with a U-III Film Camera System in Year 1 (Nikon Instruments, Inc., Melville, NY, USA) and a Konus Digital Microscope camera in Year 2 (Konus Optical and Sports Systems, Verona, Italy).

SLA was determined at 67 DAP in Year 1 and at 74 DAP and 75 DAP in Year 2. Four leaflet disks (1.2 cm^2 per disk) were sampled from each plant, dried at 60°C and weighed. A total of 72 samples were collected in Year 1 (six ILs × six plots × two plants per plot), and 60 samples in Year 2 (six ILs × five plots × two plants per plot). In Year 2, the leaflets used for leaf gas exchange measurement were saved and kept hydrated, and the disks were sampled in the laboratory. After SLA determination, the rest of each leaflet was composited back with the corresponding disk for ^{13}C and N analysis.

Chlorophyll content was determined only in Year 1. At 67 DAP, a set of four leaflet disks were frozen in liquid N and stored at −80°C until processing (a total of 72 samples). Frozen disks were crushed and 1 mL of 80% acetone was added. The mixture was shaken in a rotary shaker at 175 rpm in a dark cold (4°C) room overnight, centrifuged and diluted with supernatant 1 : 6 (v : v). Samples were read for absorbance at 470 nm, 647 nm and 663 nm wavelengths, and chlorophyll a and b determined (Lichtenthaler 1987).

Hybrid cultivars

SLA was determined from leaflets used for leaf gas exchange measurements as described above for Year 2. A total of 64 samples were collected (16 cultivars × four plots × one plant per plot). No stomatal density and chlorophyll content measurements were done.

Greenhouse study: Experimental design

A study under controlled greenhouse conditions was conducted between 2 February and 8 April 2009 (0 and 65 DAP, respectively) at UC Davis. A total of 36 11-L pots were filled with 4 kg of a modified greenhouse medium commonly used for tomato research at UC Davis (Hanan 1998). Supplemental light was provided under a day : night length cycle of 16 : 8 h. Six-week-old IL seedlings were transplanted and randomly arranged in six blocks along two benches (36 plants in total). Fertilisation was done five times with a 15–5–15 N–P–K liquid fertiliser (0.5 g N per event per pot; Peters Excel CAL-MAG Special, The Scotts Company LLC, Marysville, OH, USA) and a one-time addition of a 14–14–14 N–P–K slow release fertiliser at 11 DAP (1.1 g N per pot; Osmocote Classic, The Scotts Company LLC, Marysville, OH, USA). Watering occurred at least three times per week to reach a preset pot weight corresponding to just below the pots' water holding capacity.

Greenhouse study: Leaf gas exchange and $\Delta^{13}C$

Leaf gas exchange measurements were taken using two field portable open flow infrared gas analysers (model 6400, LI-COR Inc., Lincoln, NE, USA) with the same settings as described for the field study. The leaf chamber areas differed between the instruments (2 and 6 cm^2); therefore, for each block, all gas exchange measurements were taken with the same instrument. Plants were flowering by the first measurements, which were at a similar developmental stage as in the field. Plants had ~15 leaves and lateral shoots were starting to develop. Measurements were taken at 41 DAP, 44 DAP and 46 DAP on well-watered plants; readings at 49 DAP on water-stressed plants were so low that the data have not been included. All measurements were done between 1100 hours and 1300 hours. The leaflets used for gas exchange measurements at 49 DAP were saved for $\Delta^{13}C$ analysis.

Greenhouse study: Leaflet morphology and nutrient content

At 64 DAP, eight disks per plant were collected for SLA determination following the same procedure as in the field study. Plants were then cut at the pot surface, and biomass separated into stem, leaves and fruits. Dried samples were weighed and analysed for total C and N as described above.

Statistical analysis

Introgression lines

Both field and greenhouse experimental designs were randomised complete block designs. Analyses of gas exchange measurements (e.g. g_s or WUE_i) were performed as repeated measures under a split-plot treatment structure. Each day was considered as a 'subplot' and the error term for the ILs (i.e. main plot) was specified as error = IL × block. To compare IL traits, the contrast statements were structured as follows: Det–Obs (M82 and IL 5–4–4) versus SemiDet–Clr (IL 5–4, IL 5–4–2 and IL 5–4–5–44), Det (M82 and IL 5–4–4) versus SemiDet (IL 5–4–1, IL 5–4, IL 5–4–2 and IL 5–4–5–44) and Obs (M82, IL 5–4–4 and IL 5–4–1) versus Clr (IL 5–4, IL 5–4–2 and IL 5–4–5–44). Analysis of variance (ANOVA) and covariance (ANCOVA) were performed using the GLM procedure of SAS ver. 9.1 (SAS Institute, Cary, NC, USA). The Shapiro–Wilk W test for normal distribution and Levene's test for homogeneity of variance were used to test that the data fulfilled the ANOVA assumptions. The data were transformed as necessary when assumptions were not met. The Tukey–Kramer honestly significant difference (HSD) test was used to determine significant differences among treatments at $P < 0.05$.

Hybrid cultivars

The experimental design was structured as a randomised complete block design. Each cultivar was considered to be a sampling unit described by the measured suite of traits during the field experiment. A descriptive discriminant analysis was performed to find the discriminant function that maximised the

difference between processing tomato cultivars that differed in the presence of leaf vein chloroplasts (Obs *vs.* Clr) (McCune and Grace 2002). A total of 64 samples were included in the analysis (16 cultivars × four replicates per cultivar). The discriminant analysis included a total of 29 variables: canopy cover (49 DAP, 77 DAP and 99 DAP), leaf gas exchange variables of four measurement runs (P_n, g_s, WUE_i, vapor pressure deficit (VpdL) and leaflet temperature; see above), SLA, $P_n \times$ SLA at 68–69 DAP, shoot and fruit biomass at harvest, HI and days to maturity for each cultivar as reported by the seed company (Table 1). Because analysis was conducted on only two groups to separate cultivars with or without leaf vein chloroplasts (Obs *vs.* Clr), the output is a single function that explains all the variance between groups. Data was analysed for multivariate outliers using the Mahalanobis distance procedure. Multivariate analyses were conducted using JMP ver. 9 (SAS Institute Inc.).

Results

Field studies: shoot growth, development and biomass

Det–Obs ILs versus SemiDet–Clr ILs

In both years, the total aboveground biomass of ILs with determinate growth habit and chloroplasts along the leaf veins (Det–Obs ILs) was >40% lower than the SemiDet–Clr ILs (Det–Obs: 187 g per plant and 381 g per plant; SemiDet–Clr: 270 g per plant and 675 g per plant, for Years 1 and 2, respectively) (Tables 2, 3; Fig. 1*a*). In Year 1, leaf biomass (93 g per plant and 182 g per plant, respectively) and stem biomass (45 g per plant and 73 g per plant, respectively) were similarly lower. The final harvest occurred at an early stage in crop development due to the late planting date, and the Det–Obs ILs had much higher fruit biomass than the SemiDet–Clr ILs (49 g per plant and 12 g per plant, respectively; 74 DAP). In Year 2, when the final sampling of biomass was done after a full crop growing period, the shoot biomass of the Det–Obs ILs was about half the amount of the SemiDet–Clr ILs (stems + leaves; 250 g per plant and 546 g per plant, respectively; 115 DAP) and total fruit biomass was similar (131 g per plant and 129 g per plant, respectively). However, the Det–Obs ILs had more harvestable fruit (121 g per plant and 78 g per plant, respectively) and less green, unripe fruit (10 g per plant and 50 g per plant, respectively) than the SemiDet–Clr ILs. Regardless of the fruit ripening pattern among the ILs, the HI was >75% higher in the Det–Obs ILs (0.34 g fruit g^{-1} total biomass) than the SemiDet–Clr ILs (0.19 g fruit g^{-1} total biomass; Fig. 1*a*).

The ILs with determinate growth and leaf vein chloroplasts (Det–Obs) had a more compact canopy that covered less soil surface than the SemiDet–Clr ILs by the end of the experiments (Year 1: 64 DAP and Year 2: 77 DAP, 99 DAP and 108 DAP). Early in both seasons, there were no significant differences in soil canopy cover (45 DAP and 49 DAP for Years 1 and 2, respectively), nor in the percent of light intercepted per plant in Year 1 (data not shown). But at 64 DAP for Year 1 and 77 DAP onwards for Year 2, the Det–Obs ILs had smaller canopies and covered ~30% less area per plant than the SemiDet–Clr ILs (Fig. 1*b*). In Year 1, the amount of light intercepted per plant was also lower (42% and 65% of light intercepted m^{-2}, respectively). However, no significant differences in light interception were found among the ILs when the percent of

Table 2. Aboveground biomass of different plant organs (g per plant) as evaluated for Years 1 and 2 in the tomato introgression line (IL) field studies

IL traits were a determinate (Det) or semideterminate (SemiDet) growth habit, and the presence (Obs) or absence (Clr) of leaf vein chloroplasts. The comparisons of trait(s) by contrast statements show the *P*-values. In the trait comparison by contrast statements, lower or higher differences are shown with respect to the first trait(s). Values are mean ± s.e. Means followed by different letters are significantly different at $P < 0.05$. ns, no significant differences

Tomato lines	Growth habit	Leaf vein	Biomass (g per plant)									
			Leaf Year 1		Stem Year 1	Shoot Year 2	Fruit Year 1	Green fruit Year 2		Harvestable fruit Year 2		Total fruit Year 2
M82	Det	Obs	110 ± 16cd		53 ± 6bc	252 ± 12b	58 ± 13a	9 ± 4b		129 ± 3a		139 ± 6a
IL 5–4–4	Det	Obs	75 ± 5d		37 ± 4c	249 ± 18b	41 ± 8ab	10 ± 1b		113 ± 22ab		123 ± 21a
IL 5–4–1	SemiDet	Obs	133 ± 10bc		54 ± 5b	569 ± 71a	3 ± 2b	44 ± 8a		33 ± 11c		77 ± 13a
IL 5–4	SemiDet	Clr	177 ± 9a		78 ± 3a	480 ± 62a	8 ± 3b	36 ± 6ab		60 ± 8bc		96 ± 11a
IL 5–4–2	SemiDet	Clr	198 ± 17a		77 ± 10a	658 ± 47a	12 ± 7b	65 ± 11a		82 ± 13abc		147 ± 23a
IL 5–4-5–44	SemiDet	Clr	172 ± 17ab		64 ± 3ab	500 ± 34a	17 ± 4ab	51 ± 11a		94 ± 22ab		145 ± 29a
Contrasts												
Det–Obs *vs.* SemiDet–Clr			Lower	$P < 0.0001$	Lower	$P < 0.0001$	Higher	$P = 0.0001$	Lower	$P < 0.0001$	Higher	$P = 0.0072$	ns	$P = 0.7829$
Det *vs.* SemiDet			Lower	$P < 0.0001$	Lower	$P < 0.0001$	Lower	$P < 0.0001$	Higher	$P < 0.0001$	Lower	$P < 0.0001$	Higher	$P = 0.0003$	ns	$P = 0.2749$
Obs *vs.* Clr			Lower	$P < 0.0001$	Lower	$P < 0.0001$	Lower	$P < 0.0001$	Higher	$P = 0.0173$	Lower	$P < 0.0001$	ns	$P = 0.5206$	ns	$P = 0.3483$

Table 3. Effects of growth habit and leaf-vein chloroplast traits on biomass accumulation, soil canopy cover, canopy light interception and leaf gas exchange parameters analyzed in a contrast statement for the introgression line (IL) traits in year 1 and 2 of the field studies

IL traits were determinate (Det) or semi-determinate (SemiDet) growth habit, and the presence (Obs) or absence (Clr) of leaf-vein chloroplasts. Lower or higher differences are with respect to the first trait/s of the comparison; *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$; and '↑' indicates a trend towards a higher mean ($p < 0.10$). n.s. = no significant differences

		Growth habit and leaf vein traits of introgression lines (ILs)					
		Det-Obs vs SemiDet-Clr		Det vs SemiDet		Obs vs Clr	
Total aboveground biomass	Year 1	187 ± 16.1	270 ± 13.7***	187 ± 16.1	250 ± 13**	188 ± 11.45	270 ± 13.74***
(g plant^{-1})	Year 2	381 ± 19.7	675 ± 41.1***	381 ± 19.7	667 ± 35.7***	469 ± 40.87	675 ± 41.14***
Soil canopy cover[A]	Year 1	26 ± 2	44 ± 2***	26 ± 2	42 ± 2***	29 ± 2	44 ± 2***
(% m^{-2} plant^{-1})	Year 2	45 ± 1	64 ± 2***	45 ± 1	63 ± 2***	49 ± 2	64 ± 2***
Canopy light interception	Year 1	41 ± 3	65 ± 3***	41 ± 3	61 ± 2***	45 ± 3	65 ± 3***
(% m^{-2} plant^{-1})	Year 2	–	–	–	–	–	–
Photosynthetic rate P_n	Year 1	29.6 ± 0.7	27.6 ± 0.5*	29.6 ± 0.7	28.3 ± 0.5 ↑	29.8 ± 0.5	27.6 ± 0.5*
(μmol m^{-2} s^{-1})	Year 2	30.6 ± 0.2	29.3 ± 0.2**	30.6 ± 0.2	29.5 ± 0.2**	30.4 ± 0.2	29.31 ± 0.2**
Stomatal conductance (g_s)	Year 1	1.06 ± 0.06	0.84 ± 0.04**	1.06 ± 0.06	0.88 ± 0.04**	1.04 ± 0.05	0.84 ± 0.04**
(mol m^{-2} s^{-1})	Year 2	1.12 ± 0.03	0.96 ± 0.02***	1.12 ± 0.03	0.99 ± 0.02***	1.11 ± 0.03	0.96 ± 0.02***
Intrinsic water use efficiency (WUE_i)	Year 1	32.6 ± 1.3	38.7 ± 1.3**	32.6 ± 1.3	37.8 ± 1.1**	33.5 ± 1.1	38.7 ± 1.3**
(μmol-CO_2 mol-H_2O^{-1})	Year 2	30.0 ± 0.9	33.82 ± 0.8***	30.0 ± 0.9	33.1 ± 0.7***	30.3 ± 0.7	33.8 ± 0.8***
^{13}C discrimination	Year 1	21.48 ± 0.2	20.61 ± 0.1***	21.48 ± 0.2	20.72 ± 0.1***	21.33 ± 0.1	20.6 ± 0.1***
(Δ)	Year 2	21.82 ± 0.1	21.14 ± 0.1***	21.82 ± 0.1	21.23 ± 0.1***	21.72 ± 0.1	21.1 ± 0.1***
P_n per mass basis (P_n * SLA[B])	Year 1	688 ± 17	561 ± 11***	688 ± 17	591 ± 11**	685 ± 13	561 ± 11***
(nmol g^{-1} s^{-1})	Year 2	738 ± 7	689 ± 7 *ns*	738 ± 7	698 ± 6 ↑	735 ± 7	689 ± 7 ↑
Photosynthetic N use efficiency (P_n N^{-1})	Year 1	13.0 ± 0.3	9.9 ± 0.2***	13.0 ± 0.3	10.5 ± 0.2***	12.8 ± 0.3	9.9 ± 0.2***
(μmol g-N^{-1} s^{-1})	Year 2	17.8 ± 0.2	15.5 ± 0.1***	17.8 ± 0.2	16.0 ± 0.1**	17.7 ± 0.1	15.5 ± 0.1***

[A]Canopy cover at 65 DAP and 77 DAP for Years 1 and 2, respectively.
[B]Specific leaf area.

light intercepted per plant was analysed with canopy cover as a covariate (ANCOVA). In fact, the ANCOVA showed that 85% of the variation in light interception by the ILs can be attributed to differences in soil canopy cover per plant.

Individual traits of ILs

When the ILs were compared through a contrast statement based on their individual traits, (Det *vs.* SemiDet growth habit and Obs *vs.* Clr leaf veins), there was lower shoot biomass (leaves + stems) and total aboveground biomass, lower soil canopy cover for Year 1 (64 DAP) and Year 2 (from 77 DAP until harvest), and lower light interception in Year 1 (Tables 2, 3) in Det than in SemiDet ILs, and in Obs than in Clr ILs. The results were similar to those described above for the Det–Obs versus SemiDet–Clr contrast (Tables 2, 3; Fig. 1*a*, *b*). Similarly, total fruit biomass was higher in Year 1 (premature harvest) and similar in Year 2 (normal harvest). There was less green fruit in the Det and the Obs ILs. Harvestable fruit was higher only in the Det ILs, but was not different between the Obs and Clr ILs. Shoot and total biomass of the only SemiDet–Obs IL was intermediate between the highest and lowest values observed for all ILs, with the exception of fruit biomass, which was at the lower end of the range of values as reflected by the lowest HI in Year 2 (Fig. 1*a*). The soil canopy cover and the light interception of the SemiDet–Obs IL also had intermediate values (Fig. 1*b*).

Field studies: leaf gas exchange and WUE

Det–Obs ILs versus SemiDet–Clr ILs

In both years, leaf gas exchange data, averaged across all dates, showed that ILs with a determinate growth habit and chloroplasts along the leaf veins (Det-Obs ILs) had 7% and 4% higher P_n, 26% and 16% higher g_s, and 16% and 11% lower WUE_i than SemiDet–Clr ILs in Years 1 and 2, respectively (Table 3; Fig. 2*a–c*). The mean P_n values were 29.6 μmol m^{-2} s^{-1}and 30.6 μmol m^{-2} s^{-1} for the Det–Obs ILs and 27.6 μmol m^{-2} s^{-1}and 29.3 μmol m^{-2} s^{-1} for the SemiDet–Clr ILs in Years 1 and 2, respectively. Similarly, the mean g_s was 1.06 mol m^{-2} s^{-1} and 1.12 mol m^{-2} s^{-1} for the Det–Obs ILs and 0.84 mol m^{-2} s^{-1} and 0.96 mol m^{-2} s^{-1} for the SemiDet–Clr ILs in Years 1 and 2, respectively. As a result, the WUE_i was lower for the Det–Obs ILs (33 μmol CO_2 mol^{-1} H_2O and 30 μmol CO_2 mol^{-1} H_2O) than for the SemiDet–Clr ILs (39 μmol CO_2 mol^{-1} H_2O and 34 μmol CO_2 mol^{-1} H_2O) in Years 1 and 2, respectively. In addition, the higher leaf $\Delta^{13}C$ values of the Det–Obs ILs, an indirect measure of WUE_i, corroborated the lower WUE_i observed in the Det–Obs ILs (Year 1: 21.5 $\Delta^{13}C$ and 20.6 $\Delta^{13}C$; Year 2: 21.8 $\Delta^{13}C$ and 21.1 $\Delta^{13}C$ for Det–Obs and SemiDet–Clr ILs, respectively) (Tables 3, 4).

Individual traits of ILs

When ILs were compared through a contrast statement on single traits (i.e. growth habit or the presence of chloroplasts in leaf veins (Det *vs.* SemiDet and Obs *vs.* Clr)), P_n was higher in the Obs than in the Clr ILs in both years, but the difference between the Det and the SemiDet ILs was only significant in Year 2 (Table 3). For other measurements, the independent comparisons of the two genes showed that the Det ILs and the Obs ILs followed the same pattern as described above for the Det–Obs versus SemiDet–Clr contrast: higher stomatal conductance, lower WUE_i and higher leaf $\Delta^{13}C$, respectively (Table 3; Fig. 2*a–c*).

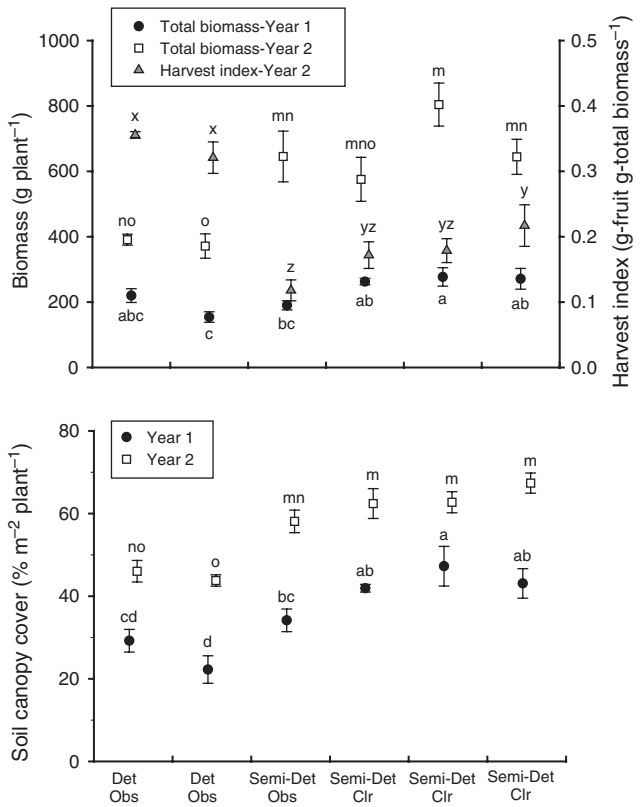

Fig. 1. Effects of tomato growth habit (determinate, Det; semideterminate = SemiDet) and leaf vein chloroplasts (presence, Obs; absence, Clr) traits on: (*a*) biomass allocation and harvest index, and (*b*) soil canopy cover of the introgression lines (ILs) for Years 1 and 2 of the field studies. ILs are listed in same order (left to right) as Table 1. Values are mean ± s.e. Means followed by different letters are significantly different at $P < 0.05$.

The mean values for the SemiDet–Obs ILs' gas exchange variables were intermediate between the values of the Det–Obs and SemiDet–Clr ILs, with the exception of P_n in Year 1, which was in the higher end of the range of values (Fig. 2*a*).

Field studies: N concentration, chlorophyll, SLA and stomatal density

Det–Obs ILs versus SemiDet–Clr ILs

The N concentration in the aboveground biomass was lower in ILs with determinate growth habit and chloroplasts along the leaf veins (Det–Obs ILs) than in SemiDet–Clr ILs (Table 5). In both years, the N content of leaflets used for gas exchange measurements was 7% lower in the Det–Obs ILs than in the SemiDet–Clr ILs. This contributed to 32% and 15% higher photosynthetic N use efficiency (P_n N^{-1}; PNUE) in the Det–Obs ILs for Year 1 and 2, respectively (Table 3; Fig. 2*d*). Chlorophyll content, which was measured in Year 1, was slightly higher (9%) in the Det–Obs ILs than in the SemiDet–Clr ILs when compared on a fresh weight basis (1.06 mg g^{-1} FW and 0.97 mg g^{-1} FW, respectively; Table 4).

The N content of leaves and stems was 18% and 15% lower, respectively, in the Det–Obs than the SemiDet–Clr ILs in Year 1 (74 DAP) (Table 5). Similarly, in Year 2, the N content in the shoots (leaves + stems) was 13% lower at 115 DAP. Given the lower aboveground biomass (Fig. 1*a*) and its lower N content (Table 5), the Det–Obs ILs had 31% and 61% less total N in the aboveground biomass in Year 1 and the shoot biomass in Year 2, respectively. In Year 1's late, short season, fruits had similar N content among all ILs, with values ranging between 3.5% and 4.5% N (data not shown).

Leaf morphology differed among ILs in Year 1 (summer planting) but not in Year 2 (spring planting) (Table 4). In year 1, SLA was 10% higher in the Det–Obs ILs than in the SemiDet–Clr ILs (22.0 $m^2 kg^{-1}$and 20.0 $m^2 kg^{-1}$ respectively; Table 4) but was similar in Year 2 (24.1 $m^2 kg^{-1}$ and 23.5 $m^2 kg^{-1}$, respectively). This implies that in Year 1, the Det–Obs ILs had thinner leaves and 23% higher C assimilation per mass basis ($P_n \times SLA$) than the SemiDet–Clr ILs (Table 4). In Year 2, $P_n \times SLA$ showed a similar trend as in Year 1 (7% increase; $P < 0.10$).

Stomatal density for all ILs was higher during Year 1 (summer planting) than Year 2 (spring planting), and differences between ILs were only observed in Year 1 (Table 4). The Det–Obs ILs had ~15% less stomata than the SemiDet–Clr ILs for both the adaxial (195 and 240 stomata mm^{-2}; Table 4) and the abaxial (288 and 342 stomata mm^{-2}) sides of the leaflets. In Year 2, all ILs had similar stomatal densities on both sides of the leaf (adaxial: 76 and 82 stomata mm^{-2}; abaxial: 151 and 154 stomata mm^{-2} for Det–Obs and SemiDet–Clr ILs, respectively).

Individual traits of ILs

The contrast analysis for single traits showed a similar pattern as described above for the Det–Obs versus SemiDet–Clr ILs. Nitrogen concentration in the shoots was lower for the Det than the SemiDet ILs, and for the Obs ILs than the Clr ILs in both years (Table 5). The leaflet N content was also lower for the Obs ILs than the Clr ILs in both years. Although the Det ILs had lower leaflet N content than the SemiDet ILs in Year 1, it was only marginally different in Year 2 ($P < 0.10$). The SemiDet–Obs ILs had N concentrations at the higher end of the range of values (Table 5). Chlorophyll content was slightly higher on a mass basis for the Det and the Obs ILs than for the SemiDet and the Clr ILs in Year 1, but there were no differences on a leaf area basis; this was not measured in Year 2. Higher SLA and lower stomatal density occurred in the Det and the Obs ILs in Year 1 (Tables 4, 5). In Year 2, these differences were not observed.

Field study: hybrid cultivars

The discriminant function to maximise the difference between cultivars either with or without chloroplasts in leaf veins (Obs *vs.* Clr) was mostly explained by leaf gas exchange measurements at the 78–79 DAP sampling, SLA and biomass at harvest (Fig. 3). In this function, the top five variables in order of relative importance and their respective standardised scoring coefficient were: P_n per mass basis ($P_n \times SLA$; –7.03), P_n (4.81), fruit biomass (–4.68), shoot biomass (4.66) and SLA (4.22). The separation of the two groups (Obs versus Clr cultivars) by the discriminant function was significant ($P = 0.03$) but not perfect, as 4 of the total 64 samples included in the analysis were misclassified (6% of the total samples; 16 cultivars × four replicate plots per

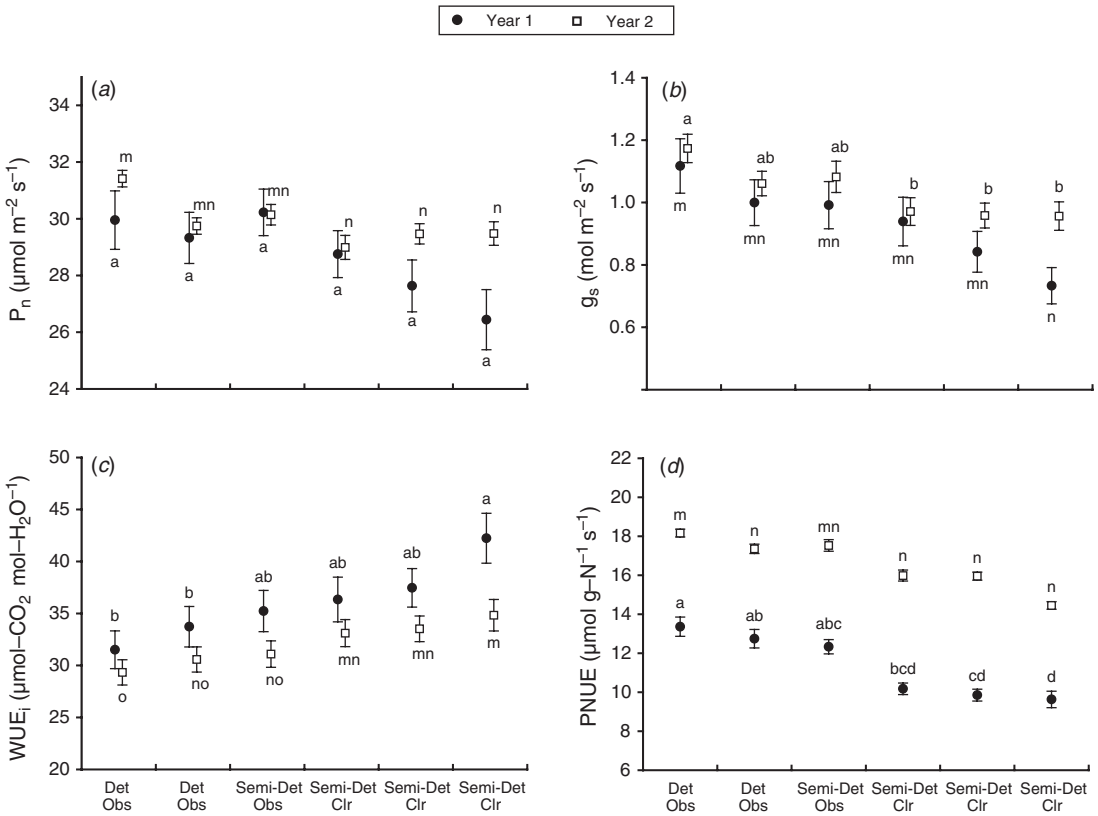

Fig. 2. Field results for Years 1 and 2 of leaf gas exchange parameters of tomato introgression lines with different growth habit (determinate, Det; semideterminate, SemiDet) and the presence (Obs) or absence (Clr) of leaf vein chloroplasts (listed in same order (left to right) as Table 1). (*a*) Leaf photosynthetic rates (P_n); (*b*) stomatal conductance (g_s); (*c*) and, intrinsic water use efficiency (WUE_i); (*d*) photosynthetic N use efficiency (PNUE). Values are mean ± s.e. Means followed by different letters are significantly different at $P < 0.05$.

cultivar). One cultivar with (Obs) and three without (Clr) leaf vein chloroplasts were misclassified in the other category by the discriminant function. The univariate ANOVAs performed to test the differences between Obs and Clr cultivars did not show significant differences. Nevertheless, Obs hybrid cultivars tended to have higher P_n and $P_n \times$ SLA than the Clr cultivars (4% and 9%, respectively, at 78–79 DAP sampling; $P < 0.10$), as seen for the individual trait contrast between Obs and Clr ILs (Table 3).

Greenhouse study

In the greenhouse, the Det–Obs ILs had lower WUE_i than the SemiDet–Clr ILs and higher $\Delta^{13}C$, which agreed with the field results (Table 6). Overall, plants in the greenhouse showed a ~20% increase in WUE_i compared with the field plants, and the difference in WUE_i between the Det–Obs and SemiDet–Clr ILs was slightly higher (19%) than observed in the field (39 μmol CO_2 mol^{-1} H_2O and 48 μmol CO_2 mol^{-1} H_2O, respectively). The total water applied during the experiment was also higher for the Det–Obs ILs (15 L *vs.* 14 L; $P = 0.003$).

Other responses were less consistent between the greenhouse and field studies. Leaf gas exchange, P_n and g_s were lower on average (a 28% and 43% decrease from the field to the greenhouse, respectively; data not shown). The Det–Obs ILs had lower P_n than the SemiDet–Clr ILs, but g_s was similar (data not shown). The growth of the SemiDet–Obs ILs was more negatively affected by greenhouse conditions than other lines, and these plants had lower biomass (data not shown), higher N concentration in leaflets and higher SLA (Table 6). As in the field, the Det–Obs ILs had lower leaflet N concentration than the SemiDet–Clr ILs. The SLA of the Det–Obs and the SemiDet–Clr ILs were similar.

Discussion

The co-location of the *obv* and *SP5G* genes appears to have had a synergistic effect that simultaneously increased P_n, decreased WUE_i and reduced shoot biomass in the Det–Obs ILs compared with the SemiDet–Clr ILs. This study suggests that a specific anatomical leaf trait (presence of chloroplasts in leaf veins) added to the suite of traits (e.g. higher P_n, SLA and fruit allocation) that contributed to gains in the yield of improved tomato cultivars. The use of ILs in this study helps to elucidate the suite of traits that accompanied the switch to cultivars with determinate growth and a one-time machine harvest in the 1960s. It is likely that an allele of the *SP5G* gene from *S. lycopersicum* contributed to a more determinate growth habit and elicited physiological, developmental and phenological changes (e.g. earlier fruit set) from the wild-type allele. The *SP5G* and the *obv* genes map to a relatively small (<3 cM) genetic region on Chromosome 5 of

Table 4. Chlorophyll content per mass basis (FW) and per unit of leaf area for Year 1, and specific leaf area (SLA), photosynthetic rate per mass basis ($P_n \times SLA$), ^{13}C discrimination and stomatal density from adaxial side for Years 1 and 2 of tomato introgression lines (ILs) leaflets from the field studies

IL traits were determinate (Det) or semideterminate (SemiDet) growth habit, and the presence (Obs) or absence (Clr) of leaf vein chloroplasts. In the trait comparison by contrast statements, lower or higher differences are shown with respect to the first trait(s). ↑, a trend towards a higher mean ($P < 0.10$); ns, no significant differences. Values are mean ± s.e. Means followed by different letters are significantly different at $P < 0.05$

Tomato lines	Growth habit	Leaf vein	Chlorophyll (mg g^{-1} FW)	Chlorophyll (μg cm^{-2})	SLA (m^2 kg^{-1})		$P_n \times SLA$ (nmol g^{-1} s^{-1})		^{13}C discrimination (Δ)		Stomatal density adaxial side (stomata cm^{-2})	
			Year 1	Year 1	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
M82	Det	Obs	1.08 ± 0.04a	32.6 ± 0.8a	22.4 ± 0.7a	24.3 ± 1.0a	700 ± 26a	763 ± 10a	21.4 ± 0.2ab	21.9 ± 0.2a	215 ± 16ab	76 ± 9a
IL 5–4–4	Det	Obs	1.05 ± 0.03ab	30.8 ± 0.9a	21.6 ± 0.9ab	24.0 ± 0.7a	676 ± 22ab	714 ± 10a	21.6 ± 0.3a	21.7 ± 0.2a	176 ± 12b	77 ± 8a
IL 5–4–1	SemiDet	Obs	0.98 ± 0.02ab	30.8 ± 0.7a	21.6 ± 0.6ab	24.1 ± 0.9a	679 ± 21ab	727 ± 13a	21.0 ± 0.3abc	21.5 ± 0.2ab	194 ± 19ab	87 ± 10a
IL 5–4	SemiDet	Clr	0.97 ± 0.02ab	30.9 ± 0.6a	19.5 ± 0.4b	24.8 ± 0.7a	571 ± 17ab	720 ± 11a	20.4 ± 0.1c	21.5 ± 0.1ab	251 ± 15a	88 ± 15a
IL 5–4–2	SemiDet	Clr	0.93 ± 0.04b	32.2 ± 0.4a	19.8 ± 0.5b	23.9 ± 0.7a	551 ± 18b	706 ± 13a	20.6 ± 0.2bc	21.1 ± 0.1bc	234 ± 15ab	72 ± 8a
IL 5–4–5–44	SemiDet	Clr	1.02 ± 0.04ab	31.3 ± 0.8a	20.8 ± 0.4ab	21.7 ± 0.8a	561 ± 23ab	640 ± 10a	20.9 ± 0.2abc	20.8 ± 0.1c	235 ± 13ab	86 ± 7a
Contrasts												
Det–Obs *vs.* SemiDet–Clr			Higher $P = 0.0035$	ns $P = 0.7253$	Higher $P = 0.0015$	ns $P = 0.3963$	Higher $P = 0.0003$	↑ $P = 0.0623$	Higher $P = 0.0002$	Higher $P < 0.0001$	Lower $P = 0.0061$	ns $P = 0.5808$
Det *vs.* SemiDet			Higher $P = 0.0027$	ns $P = 0.5215$	Higher $P = 0.0063$	ns $P = 0.4984$	Higher $P = 0.0023$	ns $P = 0.1086$	Higher $P = 0.0002$	Higher $P < 0.0001$	Lower $P = 0.0307$	ns $P = 0.4734$
Obs *vs.* Clr			Higher $P = 0.0177$	ns $P = 0.9005$	Higher $P = 0.0008$	ns $P = 0.3469$	Higher $P = 0.0001$	↑ $P = 0.0552$	Higher $P < 0.0001$	Higher $P < 0.0001$	Lower $P = 0.0022$	ns $P = 0.8265$

Table 5. Nitrogen concentrations (%) in aboveground tomato plant organs and the total N content of the introgression lines (ILs) for Years 1 and 2 of the field studies

IL traits were determinate (Det) or semideterminate (SemiDet) growth habit, and the presence (Obs) or absence (Clr) of leaf vein chloroplasts. The comparisons of trait(s) by contrast statements show the *P*-values. In the trait comparison by contrast statements, lower or higher differences are shown with respect to the first trait(s). '↓' indicates a trend towards a lower mean ($P < 0.10$). Values are mean ± s.e. Means followed by different letters are significantly different at $P < 0.05$

Tomato lines	Growth habit	Leaf vein	Nitrogen (%) Leaflet		Leaf	Stem	Shoot	Total N (g per plant) Aboveground biomass	Shoot
			Year 1	Year 2	Year 1	Year 1	Year 2	Year 1	Year 2
M82	Det	Obs	5.2 ± 0.1b	4.2 ± 0.1a	3.0 ± 0.2bc	2.2 ± 0.2b	1.9 ± 0.1b	6.7 ± 0.6ab	4.7 ± 0.2c
IL 5–4–4	Det	Obs	5.3 ± 0.2b	4.1 ± 0.1a	3.0 ± 0.1c	2.4 ± 0.1ab	2.0 ± 0.0b	4.7 ± 0.4b	4.9 ± 0.3c
IL 5–4–1	SemiDet	Obs	5.5 ± 0.1ab	4.2 ± 0.2a	3.8 ± 0.1a	2.7 ± 0.1ab	2.1 ± 0.1ab	6.7 ± 0.5ab	11.8 ± 1.4ab
IL 5–4	SemiDet	Clr	5.6 ± 0.1ab	4.5 ± 0.1a	3.8 ± 0.1a	2.8 ± 0.1a	2.2 ± 0.0ab	9.1 ± 0.3a	10.5 ± 1.4b
IL 5–4–2	SemiDet	Clr	5.6 ± 0.1ab	4.4 ± 0.2a	3.6 ± 0.1ab	2.7 ± 0.1ab	2.3 ± 0.1a	9.3 ± 0.8a	15.5 ± 1.5a
IL 5–4–5–44	SemiDet	Clr	5.9 ± 0.1a	4.4 ± 0.1a	3.5 ± 0.1abc	2.6 ± 0.1ab	2.1 ± 0.1ab	8.9 ± 1.1a	10.7 ± 0.8b
Contrasts									
Det–Obs *vs.* SemiDet–Clr			Lower $P = 0.0004$	Lower $P = 0.0351$	Lower $P < 0.0001$	Lower $P = 0.0024$	Lower $P = 0.0004$	Lower $P < 0.0001$	Lower $P < 0.0001$
Det *vs.* SemiDet			Lower $P = 0.0008$	↓ $P = 0.0841$	Lower $P < 0.0001$	Lower $P = 0.0014$	Lower $P = 0.0008$	Lower $P < 0.0001$	Lower $P < 0.0001$
Obs *vs.* Clr			Lower $P = 0.0010$	Lower $P = 0.0220$	Lower $P = 0.0030$	Lower $P = 0.0199$	Lower $P = 0.0013$	Lower $P < 0.0001$	Lower $P < 0.0001$

Table 6. Intrinsic water use efficiency (WUE_i), ^{13}C discrimination values (Δ), leaflet N concentration and specific leaf area (SLA) of tomato introgression lines (ILs) in the greenhouse study

IL traits were determinate (Det) or semideterminate (SemiDet) growth habit, and the presence (Obs) or absence (Clr) of leaf vein chloroplasts. In the trait comparison by contrast statements, lower or higher differences are shown with respect to the first trait(s). ↓,a trend towards a lower mean ($P < 0.10$); ns, no significant differences. Values are mean ± s.e. Means followed by different letters are significantly different at $P < 0.05$

Tomato lines	Growth habit	Leaf vein	WUE_i ($P_n\ g_s^{-1}$)	^{13}C discrimination (Δ)	Leaflet N (%)	SLA ($m^2\ kg^{-1}$)
M82	Det	Obs	36.3 ± 2.6c	22.7 ± 0.2ab	1.7 ± 0.0d	13.4 ± 0.6b
IL 5–4-4	Det	Obs	40.9 ± 3.2c	22.7 ± 0.3ab	1.8 ± 0.0cd	13.3 ± 0.3b
IL 5–4-1	Semi-Det	Obs	40.0 ± 3.3c	22.9 ± 0.3a	2.4 ± 0.1a	17.1 ± 0.6a
IL 5–4	Semi-Det	Clr	48.6 ± 3.3ab	21.9 ± 0.2cd	2.0 ± 0.1bc	12.8 ± 0.5b
IL 5–4-2	Semi-Det	Clr	51.3 ± 3.8a	21.3 ± 0.2d	2.1 ± 0.1b	13.0 ± 0.5b
IL 5–4-5–44	Semi-Det	Clr	42.8 ± 3.2bc	22.0 ± 0.2bc	2.0 ± 0.1bc	15.7 ± 0.5a
Contrasts						
Det–Obs *vs.* SemiDet–Clr		Lower $P < 0.0001$	Higher $P < 0.0001$	Lower $P < 0.0001$	ns $P = 0.3220$	
Det *vs.* SemiDet		Lower $P = 0.0001$	Higher $P < 0.0001$	Lower $P < 0.0001$	Lower $P = 0.0045$	
Obs *vs.* Clr		Lower $P < 0.0001$	Higher $P < 0.0001$	↓ $P = 0.0848$	Higher $P = 0.0405$	

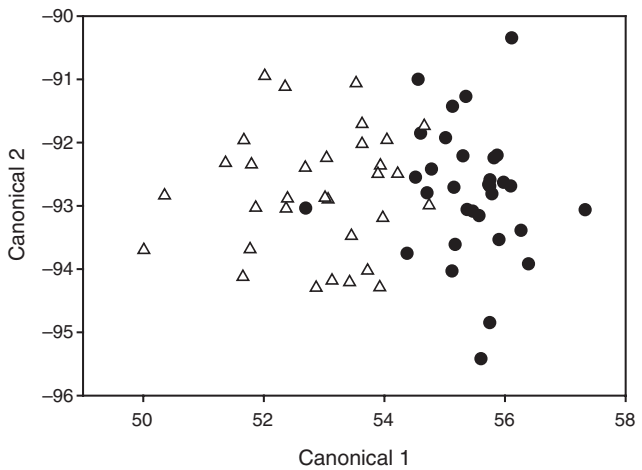

Fig. 3. Discriminant analysis plot maximising differences between the presence (Obs, ●) and absence (Clr, △) of leaf vein chloroplasts in modern processing tomato hybrid cultivars. Only one discriminant function (Canonical 1, *x*-axis) results from differentiating two groups (Obs *vs.* Clr). This function results from the descriptive discriminant analysis performed on 29 variables and represents 100% of the variance between both groups of hybrid cultivars (see Materials and methods).

tomato, which also harbours quantitative trait loci for WUE_i, plant architecture, biomass, yield and fruit quality (Eshed and Zamir 1995; Jones *et al*. 2007; Xu *et al*. 2008). Not surprisingly, the individual contributions of growth habit traits, leaf gas exchange-related traits (LeCain *et al*. 1989; Morgan *et al*. 1990; Tanaka *et al*. 2008) and the *obv* gene could not be separated using the recombinant IL 5–4 sublines available at the time of this study. Nevertheless, the results from the ILs with a determinate growth habit and leaf vein chloroplasts (Det–Obs ILs) suggest that modern cultivars with more open canopies benefited from higher P_n, partly in response to the high N fertilisation and intense light irradiances characteristic of California production systems. In addition, the reliability of abundant irrigation water supports the increase observed in g_s, which is usually associated with gains in P_n and a decrease in WUE_i (Fischer *et al*. 1998; Lu *et al*. 1998). Among highly productive processing tomato hybrids, the presence of leaf vein chloroplasts was associated with leaf gas exchange-related traits (e.g. higher $P_n \times SLA$), consistent with observations in the IL experiments. This study highlights the importance of the genetic region containing *SP5G* and the *obv* genes for future improvement of yields and WUE.

Shoot growth, development and biomass

Biomass allocation at flowering and fruit set is one of the effects associated with the *SP5G* introgression in this study. The incompletely dominant *SP5G* introgression in the determinate ILs may also contribute to earlier flowering, fewer leaves between inflorescences or a combination of both (Carmel-Goren *et al*. 2003; Jones *et al*. 2007). If the main effect of *SP5G* is on the transition from the vegetative to the reproductive stages, then no differences in canopy growth would be expected earlier in the season, as was seen in this study. Moreover, during the initial stages of plant growth, high sink demand from roots and stems, and the formation of new organs occur (Campbell *et al*. 1986); leaf demands for C can be considered secondary to other sinks (Murchie *et al*. 2009). Thus even if chloroplasts along leaf veins have a positive effect on C assimilation, the effect on productivity may have been diluted by multiple, concurrent sink demands during the vegetative stage. By harvest, the Det–Obs ILs allocated >30% of the total aboveground biomass to fruits with 54% less shoot biomass than the SemiDet–Clr ILs. Our data are consistent with a role for the *SP5G* or *obv* genes in this change in biomass allocation but do not rule out the action of other genes on the IL 5–4 introgression (Gur and Zamir 2004; Sinclair *et al*. 2004; Gur *et al*. 2010).

In the California production system, the success of a small, compact and determinate biomass allocation pattern may have been favoured by the presence of chloroplasts in leaf veins. A reduction in the total leaf biomass of Det–Obs ILs may have been partly compensated by an increase in the photosynthetic capacity of leaves with more chloroplasts near leaf veins, as will be discussed further below. This may be especially important at canopy closure when net C assimilation per plant can become

a limiting factor for higher yields (Campbell *et al.* 1986), although an increase in P_n does not always results in higher yields (Sinclair *et al.* 2004). The obscure vein trait is essentially recessive to clear veins (although chloroplast densities in the three possible genotypes have not been quantified), suggesting that the persistence of *obv* in many newer cultivars is due to its contribution towards a phenotype that performs well. This study showed a high correlation between canopy growth and light interception ($r^2 > 0.95$) in all ILs, suggesting that the reduction in shoot biomass allowed the Det–Obs ILs, with higher P_n, to allocate more C to fruits rather than to construction or maintenance of more stems and leaves per plant. These changes in biomass contributed to increase the HI of processing tomatoes, especially under a one-time mechanised harvest. Modern cultivars with a determinate growth habit and reduced light competition, as represented by the Det–Obs ILs, can be managed at higher planting densities, which contributes to yield gains per hectare when their HI is increased.

Leaf gas exchange and WUE

The observed increase in P_n and g_s in the Det–Obs ILs may be an advantage, especially under the hotter temperatures of summer plantings (e.g. Year 1) when high transpiration is required to keep lower canopy temperatures. Increases in g_s have been associated with higher-yielding cultivars of wheat and cotton (*Gossypium hirsutum* L.) (Fischer *et al.* 1998; Lu *et al.* 1998). The increase in g_s in the Det–Obs ILs was accompanied by reduced WUE_i, which was confirmed by higher $\Delta^{13}C$ in the Det–Obs ILs. Differences in WUE_i between M82 and IL5–4 have also been shown in another study (Xu *et al.* 2008); a quantitative trait locus for $\delta^{13}C$ that explains ~25% of the phenotypic expression for WUE_i in IL5–4 has been located in the same chromosome region as the *SP5G* and *obv* genes.

Leaf gas exchange traits can be purposely or inadvertently selected for, depending on how the growing conditions and other plant traits influence crop performance. Processing tomatoes are bred to be highly productive under the full sun conditions typical of California's Central Valley, which has rain-free summers with little cloud cover, and high irradiances (~2000 µmol PAR m^{-2} s^{-1}). Plants grown under higher growth irradiances may often have higher P_n (Evans and Poorter 2001), and can be accompanied by higher g_s and lower WUE_i (Tanaka *et al.* 2008). In California processing tomatoes, light saturation of the photosynthetic apparatus was not reached at 2000 µmol PAR m^{-2} s^{-1} (Bolaños and Hsiao 1991), suggesting that higher P_n in tomatoes may be achieved by adjusting the leaf composition to different light conditions (i.e. photoacclimation; Murchie *et al.* 2009). It may be speculated that the presence of leaf vein chloroplasts in the Det–Obs ILs may improve light capturing and photosynthetic activity within a leaf, resulting in higher C assimilation per leaf area.

Modern processing tomato cultivars have increased the crop WUE_a by ~50% over the last four decades (Hanson and May 2006), which coincided with the introduction of cultivars with a determinate growth habit. Growth habit has been associated with changes in traits related to leaf gas exchange and WUE_i in soybean (Tanaka *et al.* 2008) and wheat (Morgan *et al.* 1990). In tomatoes, the decrease in shoot biomass of the Det–Obs ILs could have contributed to reduce total plant transpiration, thereby increasing WUE_a. A determinate growth habit with a shorter life cycle and a relatively shorter vegetative growth phase may contribute to the reduction in the total transpiration per plant over an entire season and, consequently, the total crop evapotranspiration. In addition, the harvestable fruit biomass of the Det–Obs ILs, under the same irrigation conditions, was higher than the SemiDet–Clr ILs, indicating a gain in WUE_a and HI. Thus, in modern cultivars, the traits conferred by genes in the *SP5G* and *obv* region are likely to have contributed to maintaining total crop evapotranspiration rates similar to those observed four decades ago (Hanson and May 2006) even as planting densities increased, favouring higher yields and WUE_a at the field scale.

N concentration, chlorophyll, SLA and stomatal density

Nitrogen use efficiency (N uptake per plant biomass) of the Det–Obs ILs increased due to lower N concentration in shoots and higher HI per plant. Modern cultivars tend to have higher NUE; in wheat, this is partly associated with higher grain yields per total N in the plant (Ortiz-Monasterio *et al.* 1997). In this study, N partitioning within an entire leaf (i.e. proportion of N to leaflets) was proportionally higher to leaflets in the Det–Obs ILs than in the SemiDet–Clr ILs, although the total leaf N concentration was lower in the Det–Obs ILs. In addition, the higher P_n of the Det–Obs ILs contributed to increase the PNUE in ≥15% compared with the SemiDet–Clr ILs. PNUE can be affected by differences in the allocation of organic N towards photosynthetic compounds and further partitioning between light-harvesting complexes, electron transport and CO_2 fixation (Poorter and Evans 1998). At the plant level, the development of reproductive organs and the earlier fruit set of Det–Obs ILs may have resulted in a strong sink demand, promoting higher P_n and using leaflet N more efficiently in assimilate synthesis (Campbell *et al.* 1986; Fischer 2007; Murchie *et al.* 2009).

The SLA of all ILs was lower under the warmer temperatures of summer planting in Year 1 than in Year 2, but the Det–Obs ILs maintained a higher SLA than the SemiDet–Clr ILs in Year 1. An increase in the leaf thickness of beans (*Phaseolus vulgaris* L.) has been observed under high irradiance and temperature stress conditions, although not all varieties responded similarly (Wentworth *et al.* 2006). Among the ILs, higher SLA increased leaf P_n on a per mass basis for the Det–Obs ILs, and further increased PNUE, as has been shown for many species (Poorter and Evans 1998; Feng *et al.* 2008). Higher SLA can reduce construction costs of leaves, intercept more light per mass of tissue and increase photosynthetic N availability.

Stomatal density seems to have also been conditioned by the high temperatures at crop establishment in Year 1, affecting the leaf anatomy of future leaves (Murchie *et al.* 2009). In beans (*Phaseolus vulgaris*), an increase of up to 20-fold in stomatal density has been observed due to high temperature (Wentworth *et al.* 2006). Interestingly, g_s across all ILs was not greater in Year 1 than Year 2, regardless of the >2-fold increase in the stomatal density of the former. This agrees with the idea that the regulation of stomatal opening in mature leaves becomes more important than stomatal density *per se* (Murchie *et al.* 2009). Despite the

higher SLA of the Det–Obs ILs, the chlorophyll content of all ILs was similar on a per area basis, which may correlate with the presence of leaf vein chloroplasts in the Det–Obs ILs (Year 1). The higher chlorophyll content on a per leaf mass basis of the Det–Obs ILs suggests that with higher SLA, more chlorophyll may be directly exposed to direct light and may potentially favour higher P_n. Thus, the changes in SLA and chloroplast density on the leaf veins of the Det–Obs ILs may have had a synergistic effect on C assimilation and PNUE by increasing photosynthetic capacity with lower N per leaflet mass. It is still a challenge to relate leaf-level anatomical and morphological changes to whole-plant productivity, but results from the hybrid cultivars also point at the potential for leaf traits (e.g. SLA and the presence of chloroplasts in leaf veins) to contribute to slight increases in C assimilation and, in a more complex process, yield.

Conclusion

ILs are a valuable tool for ecophysiologists to understand how specific traits can contribute towards the adaptation of new cultivars to changing environments. We present evidence suggesting that the physiological changes that accompanied the introduction of determinate growth in processing tomatoes were favoured by gene(s) in the *SP5G–obv* region on Chromosome 5 that contribute to higher C assimilation and yield. Our results are consistent with the hypothesis that beneficial alleles of *SP5G* and *obv* were selected during the breeding of processing tomatoes, but do not definitively link these genes with the physiological traits under study. Our research also highlights the need for collective efforts by geneticists, plant breeders and physiologists to develop new cultivars adapted to higher stress conditions. This goal is facilitated by the use of exotic libraries to help identify different suites of traits (e.g. growth habit, leaf anatomy and leaf gas exchange traits) that are relevant to crop improvement.

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