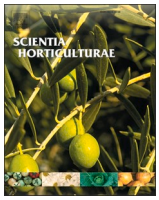
Scientia Horticulturae 323 (2024) 112497

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti



Identification of *Solanum pimpinellifolium* genome regions for increased resilience to nitrogen deficiency in cultivated tomato

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ARTICLE INFO

Keywords:
Tomato
Wild relatives
Nitrogen
NUE
Inbreeding lines
Fruit quality
Yield
Low N input

ABSTRACT

High-quality crop production with minimal fertilizer inputs is a key goal for the agriculture of the future. Globally, tomato is one of the most important vegetable crops and its intensive production and breeding has been based on the application of large quantities of nitrogen (N) fertilizers. Therefore, the development of N use efficient (NUE) cultivars with low N inputs needs to be addressed. Some variability in plant growth, fruit quality and NUE traits among tomato (*Solanum lycopersicum* L.) varieties under low N supply has been reported, however, the relevance of wild relatives of tomato has not yet been assessed. In this study, we found that *S. pimpinellifolium* accession To-937 (SP) may be a suitable resource to increase NUE in tomato. We studied a set of 29 introgression lines (IL) from SP into the Moneymaker cultivar (MM) in different seasons to investigate the potential of SP introgressions to maintain the tomato plant performance during the growth cycle under low N input in greenhouse conditions. We identified specific regions in the SP genome, on chromosomes 1, 3 and 10, involved in the responses to N inputs of fruit production and fruit quality. Notably, the line SP_10-4 maintained vegetative biomass and fruit yield production under limiting N supply. The introgressed region contained putative candidate genes as *sucrose phosphate phosphatase* (*SPP*), *invertases* (INV) and *glutamine synthase 1* (*GS1*) genes, implicated in C and N metabolism. Genomic and expression analyses revealed differences in coding and non-coding sequences as well as in mRNA levels in SP_10-4, suggesting that these genes might well contribute to the reported biomass responses to N. Additionally, line SP_1-4 showed stable fruit amino acid contents under both sufficient and limiting N supplies, indicating that assimilated N partitioning to the fruit is maintained in response to N. Altogether, our results confirmed the suitability of SP as a source of NUE related traits and the interest in the studied ILs for developing new tomato cultivars with improved NUE under sustainable fertilization conditions.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops in the world, with more than 185 million tons produced in 2020 for fresh consumption and processing (FAO, 2022). Since the Green revolution, large quantities of nitrogen (N) fertilizers have been applied to crops to obtain high yields (Lammerts van Bueren and Struik, 2017). In tomato, supplies of up to 300–400 Kg ha^{-1} of N were suggested under intensive cropping systems, but excess fertilization has been a common practice during the last decades to prevent the occurrence of nutrient deficiency (Llanderal et al., 2018). In addition, it is well known that only 50% of the nitrogen applied as fertilizer is really absorbed due to the low nitrogen use efficiency (NUE) of the otherwise high yielding crop varieties in those conditions (Truffault et al., 2019). The N fertilizer

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https://doi.org/10.1016/j.scienta.2023.112497
Received 14 June 2023; Received in revised form 4 September 2023; Accepted 9 September 2023
Available online 14 September 2023

that is not used by the plant enters the environment as N pollution. Nitrates easily leach into waterways, leading to groundwater contamination and the eutrophication of aquatic ecosystems. Water pollution as a result of excessive N fertilization in greenhouse tomato production is a particular concern in the Mediterranean region. In addition, soil nitrogen can also end up in the atmosphere as gaseous reduced N compounds, leading to troposphere pollution, and contribute to global warming (Beatty and Good, 2018). This situation has forced the implementation of regulatory policies to limit the inputs of fertilizers in agricultural fields. Therefore, one of the main goals of the agriculture industry is to make it more sustainable and environmentally friendly, while meeting food and biomass demands. For this purpose, the development of new varieties with increased NUE while using lower N inputs is crucial (Hirel et al., 2007).

NUE has been defined as the yield of a crop per unit of available N in the soil (Moll et al., 1982). It is a complex trait that encompasses several physiological processes and can be divided into the components of N acquisition, assimilation, distribution and utilization (Xu et al., 2012). Variations in NUE and related components have been identified in several crops, such as wheat, barley, legumes, oilseeds, maize, and also in the model species *Arabidopsis* (Plett et al., 2017). Nevertheless, little is known as regards the existing variability in relation to NUE in cultivated tomato. Few examples of high and low NUE genotypes have been identified and characterized up to now (Abenavoli et al., 2016; Zhang et al., 2021). It has been proposed that the contrast in NUE between the high NUE Regina Ostuni and the low NUE UC82 tomato cultivars relies on multiple physiological and molecular traits (Abenavoli et al., 2016). Among them, the processes involved in the uptake and utilization components of the NUE were highlighted, such as root length and thickness, NO_3^- influx rate, NO_3^- storage, nitrate reductase (NR) activity, root cell electrical potentials and the expression of NO_3^- transporters (Abenavoli et al., 2016; Aci et al., 2021). Nevertheless, these studies did not address the differences in yield and fruit quality parameters. In a recent study, a collection of 25 tomato genotypes was evaluated for N utilization ability to be used as rootstocks for a low nitrogen input agriculture (Zhang et al., 2021). A wide variation in N absorption capacity, biomass production and NUE was found among genotypes at different N supply levels. Interestingly, when the highest NUE genotypes were used as rootstocks, the yield was improved through induced changes in the N metabolism of the scion at low N supply (Zhang et al., 2021). A variation in organoleptic and quality profiles has also been reported in a collection of 'de penjar' tomato varieties in response to N availability (Rosa-Martínez et al., 2021).

Despite the observed variability in the processes involved in NUE in cultivated tomato, the breeding programs have focused on improving the harvest index under high N inputs, which could have reduced the presence of genetic variation controlling tomato production under low N supply. Given the large variability in N responses in the wild, one option to create new variation in NUE in tomato is to re-visit its wild ancestors (Bai et al., 2004; Li and Yan, 2020). Wild tomato relatives have been useful as a source of variation in disease resistances, fruit quality, fruit morphology and flowering time, among many other traits (Ebert and Schafleitner, 2015). Nevertheless, little attention has been paid to the selection of characters related to NUE under limited N supply.

The usability of wild alleles is difficulted by the presence of undesirable traits in the wild relatives and frequent breeding barriers. Because of this, the generation and evaluation of highly diverse introgression line (IL) populations (i.e. lines that carry a single or a few introgressions from a wild accession into an elite genetic background) facilitates the identification of new agronomically interesting alleles that can be efficiently incorporated into breeding programs (Prohens et al., 2017). Tomato ILs have been developed from different wild relative parents, including *Solanum habrochaites* S. Knapp & D.M. Spooner, *Solanum pennellii* Correll, *Solanum lycopersicoides* Dun. and *Solanum pimpinellifolium* L. (e.g. Chetelat and Meglic, 2000; Monforte and Tanskley, 2000; Finkers et al., 2007; Barrantes et al., 2014; Szymański et al., 2020). In a recent study, López-Delacalle et al. (2020) described an improved NUE in *S. lycopersicum* x *S. pimpinellifolium* Recombinant Inbred Lines (RILs) tolerant to abiotic stresses, suggesting the existence of variability in processes related to N metabolism in this wild species.

To gain insight into the performance of *S. pimpinellifolium* in the growth responses to N availability, we performed a phenotypic characterization of a *S. lycopersicum* cv Moneymaker x *S. pimpinellifolium* To-937 IL collection (Barrantes et al., 2014) including biomass, yield and fruit-quality related characters under limiting N supply over the whole life cycle. We confirmed *S. pimpinellifolium* as a suitable source of the traits involved in the processes determining the efficiency of N use for yield and fruit quality in response to N supply. Our results pave the way for the identification and characterization of NUE-related QTLs to be introduced in cultivated tomato.

2. Materials and methods

2.1. Plant material and growing conditions

A core collection (29 ILs) resulting from a cross between the To-937 accession of *Solanum pimpinellifolium* (SP) and the *S. lycopersicum* cv Moneymaker (MM) was used in this study (Barrantes et al., 2014). Each IL contains, on average, 4.25% of the SP genome, altogether covering at least 94% of the donor parent genome (Fig. S1).

Seeds were germinated in Petri dishes in the dark at 25 °C, and seedlings were transferred to trays filled with peat moss and fertilized with half-strength modified Hoagland solution (Hoagland and Arnon, 1950) containing: 8 mM N (7.5 mM NO_3^- and 0.5 mM NH_4^+), 3 mM K, 2 mM Ca, 1 mM P, 0.5 mM S and 0.5 mM Mg. Micronutrients were also provided: 16 μM Fe, 12 μM B, 5 μM Mn, 4 μM Zn, 0.5 μM Cu and 0.5 μM Mo. Three-to-four leaf plantlets were transferred to 15 L pots containing expanded clay balls (2–3 mm diameter; Arlita™, Spain) and cultivated under greenhouse conditions. The temperature ranged between 20 °C (minimum) and 35 °C (maximum). Plants were kept free from insects and diseases using standard greenhouse management procedures. The plants were vertically trained with strings, and axillary buds were removed weekly. The apical bud was removed when plants reached a height of 2.2 m (approximately 25–30 leaves), and cultivation was maintained to allow the development of the fruits until the 4th truss. Six to eight different plants of each genotype were used for each condition and sampling date in every experiment.

To determine the responses of the different ILs and parent genotypes to N availability, plants were cultivated with 8 mM nitrogen as sufficient N supply and at 4 mM nitrogen level (limiting N supply). The 4 mM N supply is compatible with a sustainable production and provokes a 50% drop in yield in the MM tomato when compared to the 8 mM N supply. These N levels were previously determined for the growth and management conditions in our greenhouses (Domínguez-Figueroa et al., 2020; Renau-Morata et al., 2021). The plants were watered daily with a fertilization solution based on Hoagland and Arnon (1950). Salts were adjusted so that the remaining essential minerals levels were unaffected.

2.2. Experimental design and sampling

In a first experiment (Exp. 2018), 12 plants of both parents (SP and MM) were grown for 6 months with differential N supply, until the fruits of the 4th trusses were developed. Both parents and the 29 ILs were grown (6 plants per genotype and N fertilization level) in a second experiment (Exp. 2019) for 2 months using differential N fertilization and the organs (leaves, stems and roots) were sampled before flowering onset (vegetative stage of growth). In the third year (Exp. 2020), both parents and the 29 ILs were grown (6 plants per genotype and N fertilization level) for 6 months (reproductive stage of growth), until the 4th truss was developed, at sufficient or limiting N supply levels. Finally, a validation assay with selected ILs, based on the observed responses to

the N supply, was performed the following year (Exp. 2021). Six plants were used per genotype and fertilization level. In every experiment, the plants were distributed in the greenhouse in rows according to the fertilization level, and the genotypes were randomly distributed within rows.

Biomass determinations (g FW and DW) were performed at the vegetative (Exp. 2019) and reproductive stages (Exp. 2018, 2020 and 2021). The roots, shoots and total fruit weights (g FW and DW) were measured. The harvest index (HI) was calculated as the ratio between the total yield and total biomass of the plant (FW). The dry organs from different plants were pooled together and homogenized in a rotor mill for analytical purposes. On each sampling date, three biological replicates per genotype and N level were used in every determination. Five representative fruits from each plant were collected for fruit quality determinations.

2.3. Carbon and nitrogen elemental analyses

The total content of elemental C and N (% DW) was determined in the sampled organs (roots, shoots and fruits) by a CN elemental analyzer (Leco TruSpec CN, Germany) at the Servicio de análisis de C y N (Estación experimental del Zaidín, CSIC, Granada, Spain)

2.4. Nitrogen accumulation efficiency determinations

Both the N accumulation efficiency (NAE) and the components of N uptake efficiency (U_N), yield N efficiency ($E_{N,Y}$) and yield biomass ($C_{N,Y}$) were determined in the Moneymaker cultivar and *S. pimpinellifolium* plants (Exp. 2018) following the methodology proposed by Weih (2014) and optimized for tomato by our group (Domínguez-Figueroa et al., 2020). The N uptake efficiency (U_N; g g^{-1}) is the ratio between mean plant N content during the main growth period and N in the seed; yield-specific N efficiency (E_N,y; g g^{-1}) is the ratio between fruit yield and the mean plant-internal N content during the main growth period; and fruit yield N concentration (C_N,y; g g^{-1}) is the N concentration in fruits. Accordingly, the overall NAE is the final N yield divided by the N content in the initial plant material, and thus the ability of crops to multiply the N available in the initial seed; and NAE = U_N x $E_{N,y}$ x $C_{N,y}$. Detailed information about the calculations performed for the determination of the NAE and its components is shown in Annex 1.

2.5. Photosynthetic determinations

Chlorophyll fluorescence measurements were taken with a MINIPAM (Walz, Germany). The effective quantum yield efficiency of PSII (PhiPS2) was determined in the 3–4th leaf from the apex as described in Renau-Morata et al. (2020). Chlorophyll contents were measured in the same leaves with a SPAD-502 (Minolta, Japan). One determination per plant was carried out and 12 measurements were taken per genotype and N supply level.

2.6. Fruit quality determinations

Metabolites contributing to taste were determined in mature red fruits, as described in Cebolla-Cornejo et al. (2012). Three representative fruits were harvested per plant from the third and fourth trusses. Three biological replicates (fruits from two different plants pooled together) were used for analyses. Sugars (glucose and fructose), organic acids (malic and citric acids) and the prominent free amino acids in tomato (aspartate, glutamate, glutamine and γ-aminobutyric acid, GABA) were quantified (mg g^{-1} FW) by capillary electrophoresis (7100 system, Agilent Technologies, Waldbronn, Germany). The total sugars (TS), the sucrose equivalents (SE) related to sweetness perception and the total prominent free amino acids (TPAA) were calculated.

2.7. Genomic and gene expression analyses of selected ILs

The expression of the selected candidate genes in the SP_10-4 introgressed region was analyzed by RT-qPCR following the procedures described in Renau-Morata et al. (2021). Analyses were performed in mature leaves. Three biological replications and three technical replicates were used for each nitrogen level (8 and 4 mM N) and genotype (MM, SP and SP_10-4). The primer pairs used for amplification are described in Y

Table S1. The *UBIQUITIN3* (*UBI3*), *clathrin adaptor complexes medium subunit* (*CAC*) and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) genes were used as the reference genes (Mascia et al., 2010; Müller et al., 2018). All selected pairs of primers were designed using Primer3 software and the In silico PCR tool of Solgenomics webpage (www.solgenomics.net), and showed similar amplicon size and amplification efficiency in *S. lycopersicum* and *S. pimpinellifolium*. The relative transcript levels of the genes were calculated following the modified ΔC_T method (Taylor et al., 2017).

Allelic variants of the SP selected genes in the SP_10-4 introgressed region among the two IL parents (MM and SP) and the reference genome SL4.0 were identified using MEGA 11 software (Tamura et al., 2021) and visualized with MView (Brown et al., 1998). Genomic SP sequences were extracted from the novo genome assembly of To-937 genome (Gayssant et al., 2022) and Moneymaker sequences inferred from the SRA SRS16871449, downloaded from https://www.ncbi.nlm.nih.gov.

2.8. Statistical analyses

Phenotypic data were analyzed by a two-way ANOVA using the Statgraphics software (Statgraphics Centurion XVI, Statpoint Tech, Inc., USA) to estimate the genetic (G), nitrogen supply (N) and interaction (GxN) effects. Discrimination among means was performed by Fisher's least significant difference (LSD) procedure ($P < 0.05$). The mean treatment values between MM and each IL were compared ($P < 0.05$) by the Dunnett test. The Pearson linear coefficients of correlations between pairs of parameters and the PCA analysis of the measured traits among genotypes were also determined by the Statgraphics software. The percentage decrease (PD) to N limitation of selected biomass parameters (PD = [(Parameter at 4 mM N – Parameter at 8 mM N)/Parameter at 8 mM N] x 100) were used in PCA analyses.

3. Results

3.1. Differential responses of biomass- and NUE-related parameters to N availability between S. lycopersicum and S. pimpinellifolium

In order to assess whether the closest wild tomato relative *S. pimpinellifolium* harbours specific alleles, involved in the responses to nitrogen limitation that can be used to improve NUE in tomato, we cultivated *S. pimpinellifolium* To-937 (SP) and *S. lycopersicum* cv Moneymaker (MM) plants in the greenhouse for 6 months under sufficient (8 mM N) and limited (4 mM N) N supply (Exp. 2018).

Biomass (g DW) and the parameters related to nitrogen assimilation efficiency were determined at the end of the experiment. The results (Fig. 1A) showed greater vegetative (shoot and root) biomass in SP than MM plants under sufficient N supply. N limitation led to a drop in biomass production in both genotypes. However, the SP plants showed higher vegetative growth at 4 mM N than MM (Fig. 1A). In contrast, the SP fruit biomass was markedly lower compared to MM (Fig. 1B). Nevertheless, the reduction in fruit biomass when N supply was limited was smaller in SP than MM (22% vs. 48%, respectively).

The analysis of nitrogen use efficiency-related parameters under sufficient N supply conditions showed greater N uptake efficiency (U_N) and fruit N content ($C_{N,Y}$) in the SP plants compared to MM (Fig. 1C,E). However, the higher partition of photoassimilates for fruit growth in MM leads to higher yield-specific N efficiency ($E_{N,Y}$) and nitrogen

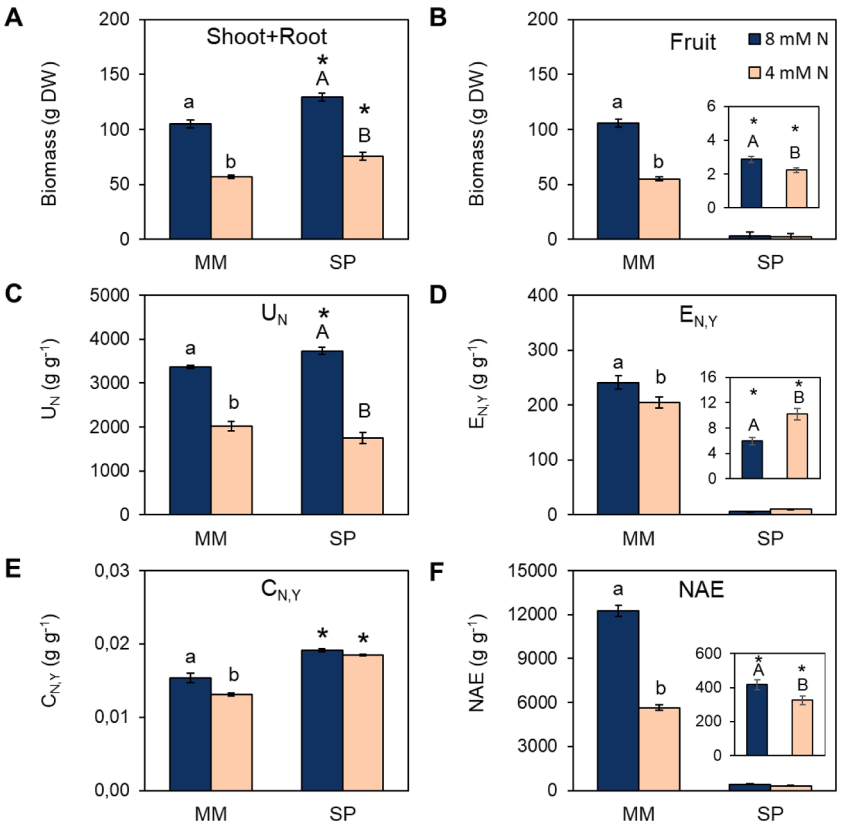

Fig. 1. Biomass and nitrogen assimilation efficiency (NAE) parameters in Moneymaker tomato (MM) and *S. pimpinellifolium* (SP) plants grown under sufficient (8 mM N; blue bars) and limiting (4 mM N; orange bars) N supply conditions. Vegetative (A) and fruit (B) biomass were determined in 6-month old plants cultivated in the greenhouse. (C) N uptake efficiency (U_N; g g^{-1}), (D) yield specific N efficiency ($E_{N,Y}$; g g^{-1}), (E) fruit N content ($C_{N,Y}$; g g^{-1}) and (F) NAE (g g^{-1}) were estimated after Weih (2014). Due to scale differences, data of SP fruit biomass, $E_{N,Y}$ and NAE are shown in the small boxes. Values are mean (±SE) of 3 determinations in 12 different plants. For each genotype and parameter, different letters indicate significant differences by effect of N level (P <0.05). Asterisks indicate significant differences between genotypes for a given parameter and N level (P <0.05).

assimilation efficiency (NAE) parameters than in the wild relative (Fig. 1D,F).

Under N limiting conditions (4 mM N), the N uptake efficiency, U_N, decreased in both genotypes and displayed similar values in SP and MM plants (Fig. 1C). In MM plants $E_{N,Y}$, $C_{N,Y}$ and NAE also decreased at 4 mM N (Fig. 1D—F). However, yield-specific N efficiency ($E_{N,Y}$) increased and fruit N content ($C_{N,Y}$) remained unaltered at 4 mM N in SP.

3.2. *Characterization of the growth responses of the IL collection to N supply during the vegetative growth stage*

The previously described results (Exp. 2018) indicated that SP exhibited greater vegetative growth under both limiting and sufficient N supplies. To identify the genetic regions involved in the observed differential growth responses to N, we cultivated the core collection (29 ILs) developed by Barrantes et al. (2014), as well as the MM and SP parents, in greenhouse conditions under the same two N concentrations (Exp. 2019).

The biomass-related parameters (dry matter and N contents of organs) and photosynthetic parameters (PhiPS2 and chlorophyll content) determined after 45 days are depicted in Fig. 2 and Table S2. The ANOVA revealed significant differences between ILs for all the traits analyzed in the vegetative stage of growth of the ILs collection (Table S2). Furthermore, the interactions (GxN) were significant (P <0.05) for every measured parameter, indicating differential responses of the ILs to the N supply.

With sufficient N supply, the average total vegetative (shoot and root) dry matter values for most of the ILs were intermediate to those of both parents (Table S2) and several ILs, such as SP_2-5, SP_10-1, SP_10-4 and SP_11-2, showed similar values to MM (Fig. 2A). Nevertheless, a group of ILs was identified as showing a greater N concentration, chlorophyll content and photosynthetic efficiency (PhiPS2) than MM (Fig. 2). The highest N contents were observed in SP_5-5, SP_6-4 and SP_12-3 ILs, as found in SP (Fig. 2B). Furthermore, SP_7-2 and SP_12-3 displayed the highest PhiPS2 values (Fig. 2C), whereas several ILs (e.g. SP_1-1, SP_2-5, SP_3-3, SP_9-4, SP_10-5 and SP_12-5) exhibited a higher chlorophyll content than parental genotypes (Fig. 2D).

The limitation in N supply provoked a drop in the biomass of most genotypes, although several ILs showed similar maximal values to those observed in MM and SP (e.g. SP_2-5, SP_6-5 and SP_11-2). The limitation in N supply provoked a reduction in N concentration in most genotypes compared to those in sufficient N supply conditions (Fig. 2B). Notably, SP_2-5 maintained N content and, most strikingly, SP_10-5 increased, demonstrating clear transgressive behavior. A high degree of variability was found in the response of photosynthetic efficiency and chlorophyll content to N limitation (Fig. 2C). Among them, SP_7-2, SP_10-3, SP_10-4 and SP_11-2 showed the best performances in response to N availability. Together, these results highlight specific regions in the SP genome in which the performance under low N supply is improved for the analyzed traits.

3.3. *Characterization of the biomass production responses to N in the IL collection during the reproductive growth stage*

Our early survey (Exp. 2018) on the growth and NAE responses of the *S. pimpinellifolium* to limited N supply indicated that the SP wild relative displayed smaller reduction in biomass production under limited N supply (4 vs 8 mM N) when compared to MM (Fig. 1). In addition, N distribution to the fruit at 4 mM N was less reduced in SP than MM, suggesting that *S. pimpinellifolium* could retain interesting traits related to N partitioning. To identify the putative genetic regions in SP involved in the use of dry matter and N for fruit growth purposes, we cultivated the IL collection in the greenhouse for 6 months to assess yield responses to N supply (Exp. 2020).

As mentioned above (Exp. 2018), MM exhibited greater fruit biomass but lower vegetative biomass than SP under sufficient N conditions at the end of the experiment (Table S2). Of the ILs, the vegetative biomass (shoot + roots) of ILs SP_2-5, SP_4-1 and SP_11-1 were also greater than

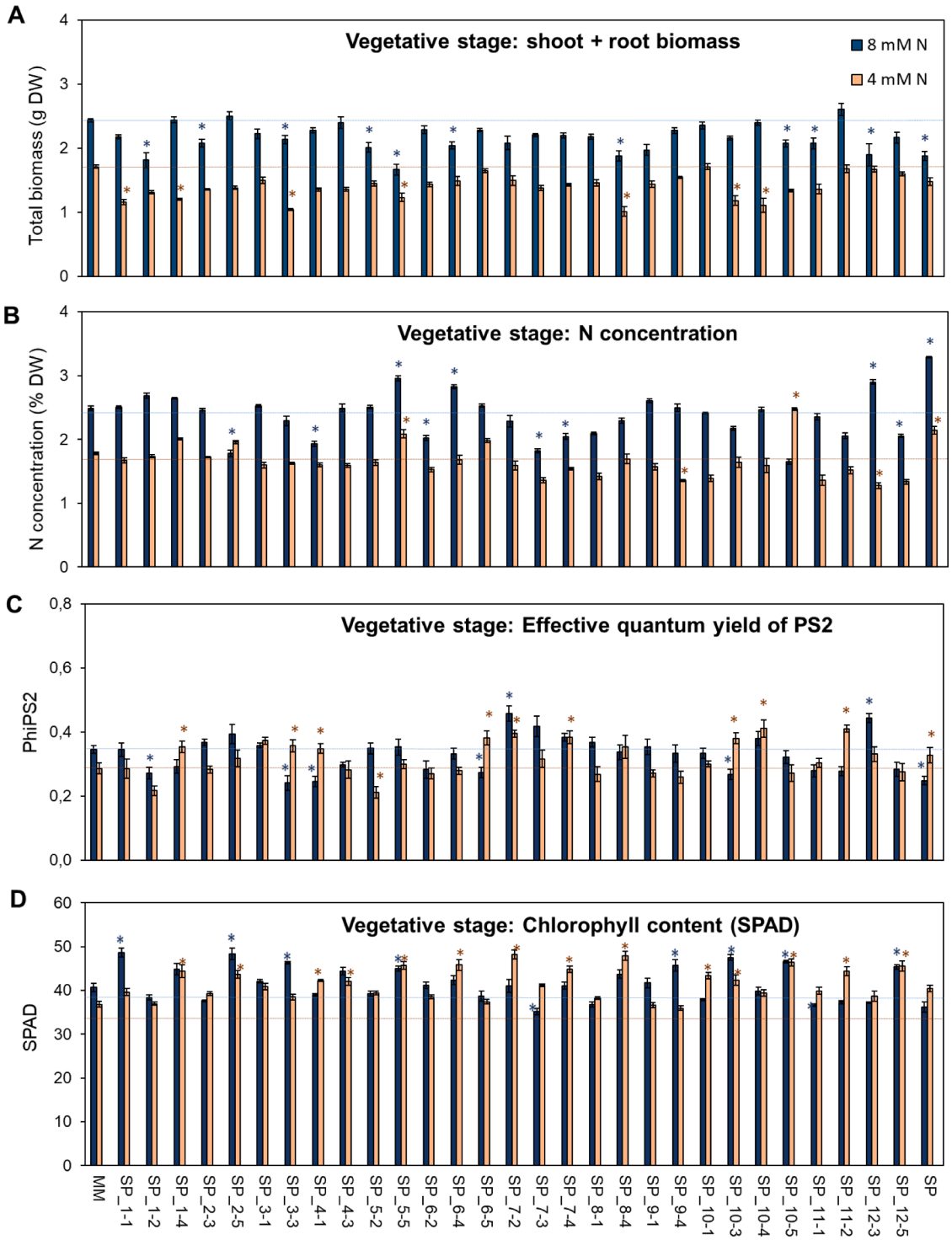

Fig. 2. Total biomass (A), nitrogen concentration (B), effective quantum yield (C) and chlorophyll content (D) in an introgression line (IL) collection of *S. pimpinellifolium* (SP) in Moneymaker tomato (MM) grown under sufficient (8 mM N; blue bars) and limiting (4 mM N; orange bars) N supply conditions during the vegetative stage of growth. Values are mean (±SE) of 6 determinations in different plants. Blue and orange horizontal lines indicate the value for MM in each parameter in 8 and 4 mM N supply conditions, respectively. For each N level, significant differences ($P < 0.05$) between each genotype and MM are indicated by an asterisk (Dunnett test).

MM (Fig. 3A). Most of the ILs exhibited lower total fruit biomass than MM. Notably, a similar amount of fruit dry matter was observed in some ILs, such as SP_3-1, SP_5-5, SP_9-4 and SP_12-5 (Fig. 3B). In relation to the N contents in vegetative biomass, several genotypes displayed a higher N concentration in the vegetative organs (e.g. SP_1-4, SP_4-3, SP_7-4, SP_10-5 and SP_12-3) compared to MM (Fig. 3C).

Under N limitation conditions, lower vegetative biomass was observed in MM and SP, as well as in several ILs (Fig. 3A) compared to control conditions. Nevertheless, some ILs showed a higher amount of vegetative dry matter than the MM parental, such as SP_2-5, SP_6-5, SP_10-4, SP_11-2 and SP_12-3. Interestingly, the vegetative biomass was similar under limiting and sufficient N levels in SP_10-4, SP_11-1, SP_11-

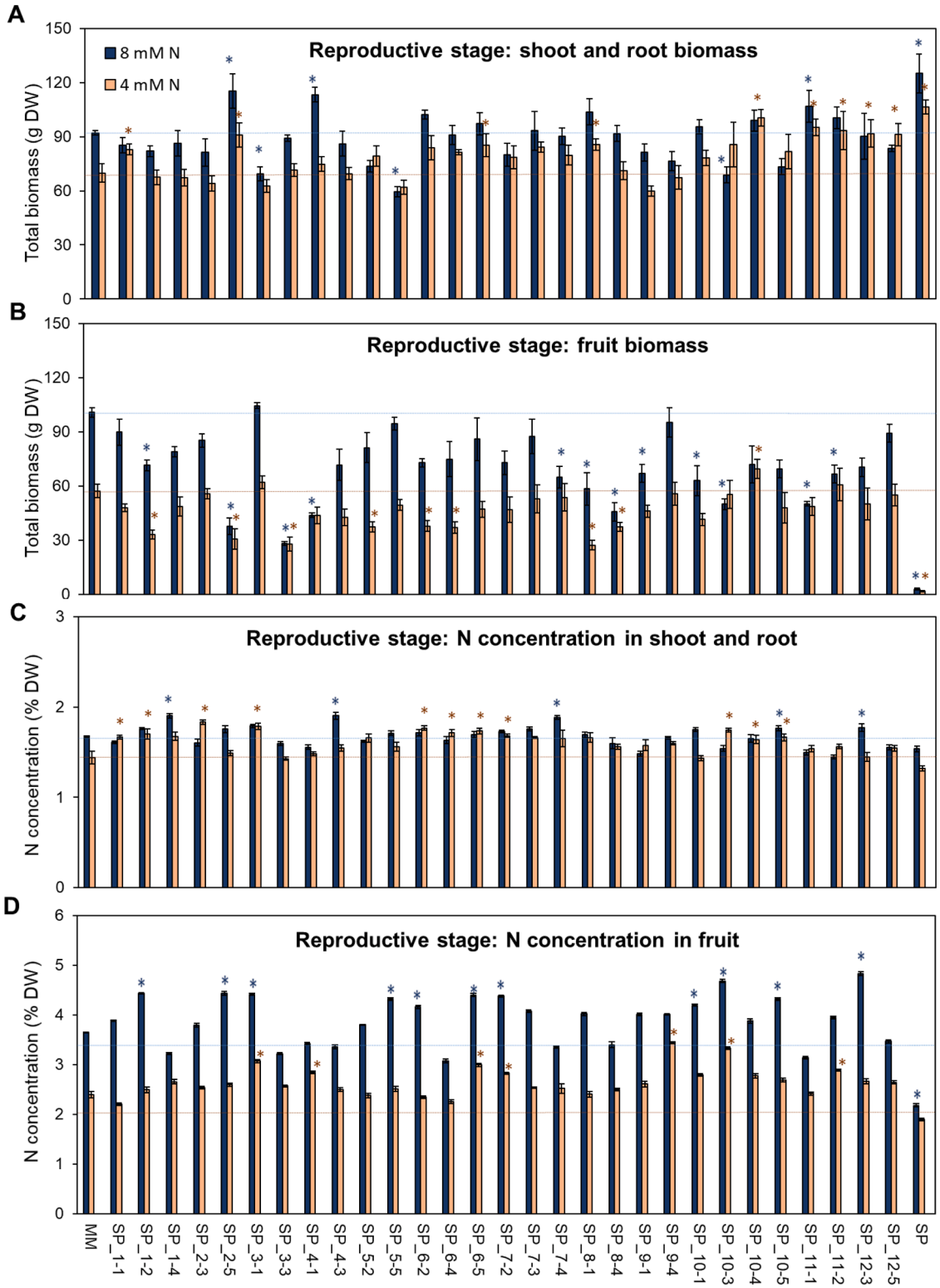

Fig. 3. Biomass (A,B) and nitrogen concentration (C,D) of vegetative and fruit organs in an introgression line (IL) collection of *S. pimpinellifolium* (SP) in Moneymaker tomato (MM) grown under sufficient (8 mM N; blue bars) and limiting (4 mM N; orange bars) N supply conditions during the reproductive stage of growth. Values are mean (±SE) of 6 determinations in different plants. Blue and orange horizontal lines indicate the value for MM in each parameter in 8 and 4 mM N supply conditions, respectively. For each N level, significant differences (P <0.05) between each genotype and MM are indicated by an asterisk (Dunnett test).

2 and SP_12-5. Total fruit biomass also dropped in most genotypes with 4 mM N supply (Fig. 3B). It must be highlighted that SP_10-4 plants exhibited no reduction and the highest total fruit biomass under N limitation conditions. Differences in the responses of the N contents in vegetative organs and fruits were also observed among the ILs under N limiting supply conditions. SP_2-3, SP_3-1, SP_6-5, SP_10-3, SP_10-4 and SP_10-5 ILs showed a higher N concentration in shoots and roots (Fig. 3C). In fruit, a higher N concentration was found in SP_3-1, SP_4-1, SP_6-5, SP_9-4 and SP_10-3 (Fig. 3D).

Altogether, we identified regions in SP involved in the production of dry matter for vegetative and fruit growth in response to N. In order to better display the partition of total plant biomass for fruit and assess the relationship with plant yield in tomato, we determined the harvest index (HI) among the ILs. Fig. S2 shows the variation in HI, indicating the changes in the proportion of total plant fresh weight as fruit between the ILs and N supply. Notably, we found that total fruit biomass displayed a close correlation with the harvest index ($r = 0.89$, $P < 0.05$), suggesting that the identification of traits related to fruit yield can be based on the selection of SP regions involved in fruit dry matter production.

3.4. Correlations among growth and fruit biomass traits

To determine the relationship between growth parameters and the effect of the developmental stage of the plants, a correlation analysis was performed in the studied IL collection. Significant ($P < 0.05$) correlations were detected among the phenotypic traits determined at both vegetative and reproductive growth stages (Fig. S3).

Under sufficient N supply conditions, the total biomass of the plant at the vegetative stage was mainly related to the shoot biomass ($r = 0.90$) and total C content ($r = 1.00$) but did not correlate with total N content. Interestingly, shoot dry matter was negatively correlated with N concentration ($r = -0.48$). When there was limiting N supply (4 mM N), the total dry matter of the plant depended both on shoot biomass ($r = 0.93$) and root biomass ($r = 0.71$). It is noteworthy that the ILs with the highest total C content corresponded to those with the highest total N content ($r = 0.53$), and thus, total biomass was related to N content under limiting N supply conditions.

At the reproductive stage and with sufficient N supply (8 mM N), although the ILs with the highest total biomass were those with the highest fruit biomass ($r = 0.76$), no correlation was found between total and vegetative (shoot +root) biomass (Fig. S3). In addition, fruit biomass was correlated with total N in fruits ($r = 0.94$), as well as vegetative biomass with the N content ($r = 0.90$). Although N concentration contributes to the total N in fruits ($r = 0.66$), total N content in the vegetative biomass was not correlated with the N concentration in shoots and roots (NS). In relation to C and N, total C and N contents were correlated in shoots and roots ($r = 0.82$) and fruits ($r = 94$). When N supply is limited at the reproductive stage, the total biomass of the plant depended on both vegetative and fruit biomass ($r = 0.60$ and 0.68, respectively). In addition, the total N accumulated by the plant, although mainly dependent on the N partitioned to the fruits ($r = 0.92$), was also correlated with the total N in the shoots and roots ($r = 0.44$). With 4 mM N supply, the total C and N in fruits were closely related ($r = 0.95$), but this correlation decreased ($r = 0.59$) in shoots and roots, when compared to sufficient N supply.

It has to be noted that the total fruit biomass and total N in the fruits, parameters related to yield, were not significantly correlated with the growth parameters determined during the vegetative stage of growth (Fig. S3). This lack of correlation was observed both under sufficient (8 mM N) and limited (4 mM N) N supply conditions. The only correlation found was a weak, but significant one, between total plant biomass at vegetative and reproductive stages ($r = 0.41$) under sufficient N supply (8 mM N) conditions.

In order to assess how the ILs are grouped according to their responses to the reduction in N supply, a PCA analysis was conducted. For this analysis, the percentage decrease (PD) in the selected growth parameters (biomass and N contents) under limited N supply (4 mM N) conditions compared to sufficient (8 mM N) conditions was used (Fig. 4 and Fig. S4). The three principal components (PCs) of the PCA explained 69% of the total variation observed, with PC1, PC2 and PC3 accounting for 32.4%, 19.1% and 17.3% of the total variation, respectively. The plot including PC1 and PC3 most clearly displays the differences between the studied ILs (Fig. 4). The PD of fruit dry matter at the reproductive stage was the parameter displaying the greatest correlation with PC1, while the PD of shoot and root dry matter at the vegetative stage were the ones with the greatest negative correlation (Fig. 4). Moreover, the PD of N concentration in shoots and roots and fruit during the reproductive stage exhibited the greatest correlation with PC3, whereas the PD of N concentration in shoots and roots at the vegetative stage was the lowest. The PCA plot including IL and parents showed that SP_10-5 and SP_2-5 were the farthest, and with highest PD values in N concentrations and biomass during the vegetative stage (Fig. 2). We highlighted the fact that SP_1-4, SP_4-1, SP_10-3, SP_10-4 and SP_11-2 (with negative values for PC1 and negative for PC3) displayed the smallest decreases in fruit biomass and N concentration in the fruit under N limitation conditions when compared to when there was sufficient supply, as well as the smallest decreases in shoot and root biomass at the reproductive stage (Fig. 3). Of these, we found SP_10-4 of special interest since no decrease in shoot, root or fruit biomass at the reproductive stage under N supply limitation conditions was observed (Fig. 3A-B).

3.5. Impact of N limitation on fruit quality traits in the IL collection

Since N availability has an impact on the contents of major organic compounds related to fruit quality in tomato (Renau-Morata et al., 2021), we measured the contents in sugars, organic acids and amino acids, which contribute to the organoleptic properties of the fruits (Exp. 2020). The donor parent, SP, presented a marked accumulation of soluble solid content (SSC), specifically of sugars and citric acid, compared to MM, while the derived ILs resembled, in general, the recurrent parent (Table S3). No differences were found, though, between the parent lines as regards the accumulation of malic acid and free prominent amino acids.

Under our assay conditions (Exp. 2020), the composition of SP fruits did not change with limiting N supply, and the sugar and acid profile of the fruit remained unaltered (Fig. 5). However, in the case of MM and the ILs, limiting doses of N led to a reduced accumulation of prominent amino acids, with the GABA, glutamine and glutamic acid contents being more affected than aspartic acid (Fig. 5, Table S4). Of the ILs, three interesting cases were identified. SP_3-3 presented a higher accumulation of amino acids, specifically aspartic and glutamic acids, than the fruits of the recurrent parent and other ILs when grown with normal N supply, but this outstanding performance was not maintained under N limiting supply conditions. On the other hand, SP_1-4, SP_10-5 and SP_9-1 tended to show a greater free amino acid accumulation both under sufficient and limiting conditions (Fig. 5; Table S4). This effect was mainly due to a bigger accumulation of glutamine and glutamic acid in most cases, being more evident under limiting conditions; and not a consequence of fruit size. Nonetheless, these effects remained close to the signification level but above the 0.05 threshold (Table S3). Another group of lines, including SP_1-1, SP_6-2 and SP_10-1, did not outstand for highest amino acid contents, but they did not show a reduction in the accumulation of amino acids under limiting N supply (Fig. 5, Table S4) conditions. This performance resembles that of the SP, which also maintained a similar accumulation under normal and limiting N supply conditions.

Regarding sugars and organic acids, their accumulation did not increase in the ILs as a result of SP introgression (Table S4). On the other hand, some of them tended to show reduced contents, though this trend was not consistent in both growing conditions. Only SP_6-4 presented a reduced malic acid accumulation both under normal and limiting N-supply conditions when compared to both parents.

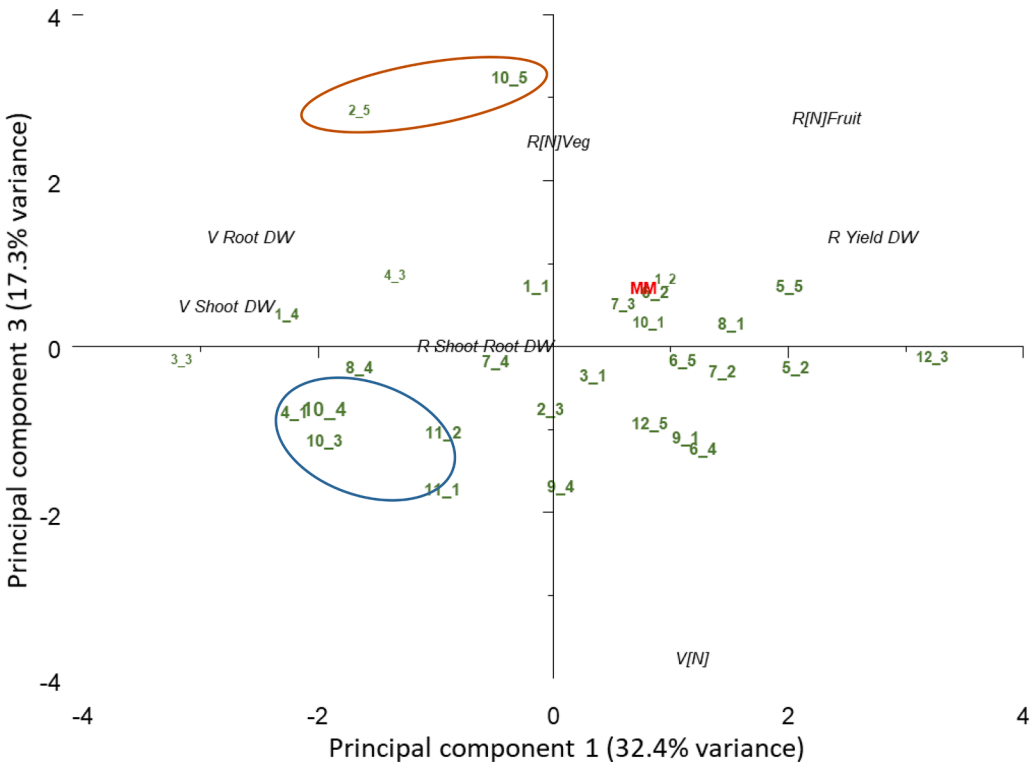

Fig. 4. PCA loading plot and score plot for the percentage decrease (PD) in biomass and N use parameters between limiting and sufficient N supply conditions for the IL collection and the parents (Experiment 2020). The first and third components were displayed. Red letters: MM parent; Green letters: ILs. Acronyms used of the PD between limiting and sufficient N supply were: R[N]Fruit: N concentration in fruits at reproductive stage; R[N]Veg: N concentration in shoots and roots at reproductive stage; R Yield DW: dry matter in fruit at reproductive stage; R Shoot Root DW: dry matter in shoots and roots at reproductive stage; V Root DW: root dry matter at vegetative stage; and V Shoot DW: shoot dry matter at vegetative stage. Selected ILs in the text were grouped by ellipses.

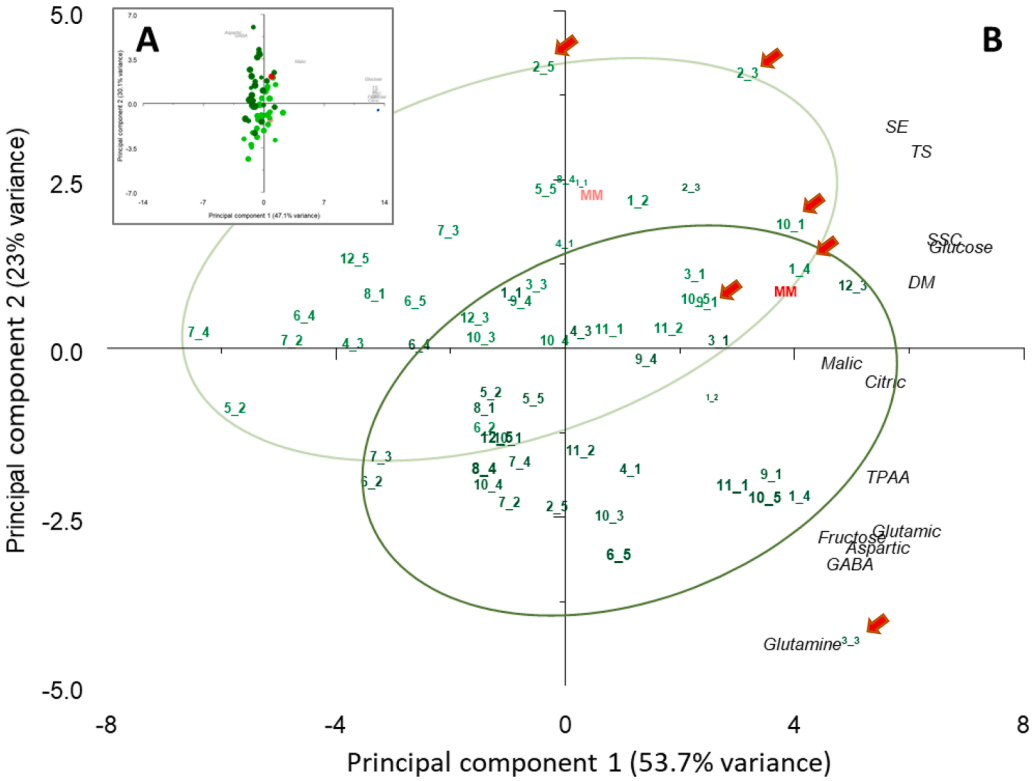

Fig. 5. Principal component analysis biplot of fruit composition from plants of the IL collection grown under sufficient (8 mM N; darker color) and limited (4 mM N; lighter color) conditions. The first and second components were displayed. A) Including *S. pimpinellifolium* parent (blue dot). B) Excluding *S. pimpinellifolium* parent. Dot and font sizes are proportional to fruit weight. Acronyms used were TS: total sugars; SE: sucrose equivalents; TPAA: Total prominent amino acids. Dark and light green ellipses group the ILs for sufficient and limiting N supply conditions, respectively. Selected ILs in the text were identified with red arrows.

3.6. Validation of the identified ILs for plant performance under N limitation conditions

In order to verify the genetic effects observed in the previous experiments, we cultivated MM and SP_1-4, SP_3-1 and SP_10-4 in the greenhouse the following year to assess fruit biomass and quality parameters in response to N supply (Exp. 2022). SP_3-1 and SP_10-4 were selected for their biomass responses to limiting N supply conditions, while SP_1-4 was chosen for the amino acid content in fruit under limiting N supply (Figs. 3 and 5) conditions. Vegetative and fruit

biomass production are shown in Fig. S5. Interestingly, fruit biomass responses to N availability were similar to those observed in the characterization experiment of 2020 (Fig. 3). Notably, no decrease in fruit biomass with limiting N supply was observed in SP_10-4 when compared to conditions of sufficient supply (Fig. S5), as reported in the former assay (Exp. 2020). Furthermore, parameters related to the organoleptic quality of the fruit were also determined in fruits of SP_1-4, SP_3-1 and SP_10-4, and the sugars, organic acid and amino acid contents were measured. ILs and MM were observed to have similar contents of organic acids and sugars, as shown in the first experiment (Table S4). It is noteworthy that SP_1-4 confirmed the greater accumulation of glutamine, glutamate and aspartate (Fig. S6) when compared to MM. Furthermore, a similar trend in the fruit biomass and organoleptic-related parameters was observed in MM, SP_1-4, SP_3-1 and SP_10-4 in the two independent surveys (Exp. 2020 and 2022). Altogether, these results validated the genetic effects observed previously.

3.7. Identification of candidate genes involved in the responses to N in the SP_10-4 introgressed region

To further investigate the genetic basis of the effects observed in SP_10-4, we analyzed the introgressed chromosomal region. Since SP_10-4 and SP_10-5 showed overlapping regions in chromosome 10 (Fig. S1), but the phenotypic response to N limitation was not observed in SP_10-5, we inferred that the genes responsible for the phenotypic behavior of SP_10-4 should be localized inside the specific region of SP_10-4 introgression (SL4.0ch10:61800664…62590355). A search throughout the tomato reference genome (SL4.0 version and ITAG4.0 annotation; https://solgenomics.net) showed, among others, four candidate genes involved in C/N metabolism in the introgressed region which might be related to the growth and biomass responses observed (Table S4; Fig. S7): the *invertase 6* (Solyc010g083290.4.1) and *invertase 8* (Solyc10g083300.2.1) genes, the *sucrose phosphate phosphatase* (Solyc10g081660.2.1) and the gene for *glutamine synthetase 1* (Solyc10g083550.1.1). Invertases (INV) are enzymes which catalyze the cleavage of sucrose into fructose and glucose and have been related to the activity of sources and sinks in different crops (Kingston-Smith et al., 1999; Li et al., 2012). Sucrose phosphate phosphatase (SPP) catalyzes the final step of sucrose in the pathway of sucrose synthesis, and together with sucrose phosphate synthase promotes plant growth and biomass accumulation (Maloney et al., 2015). Glutamine synthetase 1 (*GS1*) is key in plant nitrogen assimilation and recycling (Bernard and Habash, 2009).

The comparison of the sequences of the candidate genes between the SP (Gayssant et al., 2022), MM and the tomato reference genome (Heinz, https://solgenomics.net, Fig. S8) showed one non-synonymous mutation (exon 3) in the *invertase 6* gene and three non-synonymous mutations (exons 1 and 4) in the *invertase 8* gene (Table S5; Fig. S8) between both cultivated genomes and the wild genome. In the case of *glutamine synthase 1* (*SlGS1*) gene three non-synonymous mutations were identified in SP compared to cultivated tomato genomes (Table S5; Fig. S8). However, a deletion of two base pairs was also observed in MM, likely causing a frameshift mutation. Only two synonymous changes were found in the *sucrose phosphate phosphatase* gene among wild and cultivated genomes. Together, these non-synonymous changes may have a role at the functional level in the identified alleles, and thus, might be related to the phenotypic traits observed in the SP_10-4. In addition, we also observed differences in the promoter regions of the candidate genes in SP sequences when compared with MM and Heinz (Table S5; Fig. S8) that might have an impact on the expression of the candidate genes.

In order to determine whether the mRNA levels of the candidate genes might also contribute to the biomass and yield responses to N observed, we analyzed the expression of *GS1, INV6, INV8* and *SPP* genes by RT-qPCR in SP_10-4, SP and MM under N limiting (4 mM N) and sufficient (8 mM N) N supply conditions. A drop in the expression levels of both *GS1* and *SPP* genes in MM and SP leaves was observed when N availability was limited from 8 to 4 mM (Fig. 6A,B). Interestingly, *GS1* and *SPP* mRNA levels increased in SP_10-4 leaves at 4 mM when compared to 8 mM. Furthermore, at limiting N supply, SP_10-4 showed higher *GS1* and *SPP* expression levels than MM and SP, suggesting improved performance of N metabolism and sucrose synthesis processes, respectively.

On the other hand, the expression of *invertase 6* was not affected by N levels in MM and SP leaves, but a slight increase at 4 mM N was observed in SP_10-4 (Fig. 6C). Slight changes were observed in mRNA levels of *invertase 8* gene in SP and MM leaves under 4 mM N compared to 8 mM (Fig. 6D), but not in SP_10-4.

4. Discussion

4.1. S. pimpinellifolium as a source for traits involved in the growth responses to N supply

In this study, we used an IL population of *S. pimpinellifollium* genomic fragments in the genetic background of fresh marker tomato Moneymaker (Barrantes et al., 2014) in order to evaluate the effect on the growth and agronomic responses in different N supply conditions. *Solanum pimpinellifollium*, the closest wild ancestor to cultivated tomato, has previously been used for the introduction of disease and pest resistance and abiotic stress tolerance (Bai et al., 2004). Furthermore, several additional traits, which have breeding potential, have been identified in SP, such as fruit sugars, soluble solids and lycopene contents, fruit weight and shape, shelf life, yield and plant growth habit (Capel et al., 2015; Ebert and Schafleitner, 2015; Di Giacomo et al., 2020). However, to the best of our knowledge, there are no reports on the use of SP as a source of variability for traits related to the improvement of NUE. Only a study by López-Delacalle et al. (2020) reported an efficient N metabolism in two RILs from a cross between *S. lycopersicum* CLN2498E and *S. pimpinellifolium* LA1579 when subjected to combined salinity and heat stress.

Our survey revealed that the closest wild relative to tomato retains interesting genes for traits involved in biomass production and nitrogen use efficiency (NUE) parameters. SP displayed greater N uptake efficiency and higher N concentration in fruits under sufficient N fertilization conditions. Moreover, under limited N supply SP also increased the yield-specific N efficiency, a parameter related to the N partitioning for fruit development (Domínguez-Figueroa et al., 2020). Accordingly, the N concentration remained unaltered in the fruits of SP plants grown at 4 mM N. These results indicate that SP shows an improved capacity to maintain the partition of N to the fruits when the availability of this element is restricted. It is worth noting that the reduction in N supply in the limiting treatment of this study is compatible with sustainable tomato production (Renau-Morata et al., 2021).

Altogether, these traits are of great interest for use in breeding programs aimed at the development of tomato cultivars with increased NUE and fruit nutritional quality under limiting N fertilization conditions. Although similar strategies have been successfully addressed for the purposes of improving NUE in several extensive crops, such as maize (Coque and Gallais, 2007), rapeseed (Abdel-Ghani et al., 2013), rice (Shen et al., 2021) or wheat (Sandhu et al., 2021), much less research has been carried out into vegetables (Villanueva et al., 2021).

4.2. Characterization of growth and yield-related responses to N of the ILs library

The IL collection of *S. pimpinellifollium* used in this study has previously been evaluated for traits and characteristics related to fruit quality under sufficient N supply conditions (Barrantes et al., 2016). Interestingly, characters involved in the determination of fruit weight and plant vigor were also identified. Accordingly, phenotypic diversity for plant fresh weight was described in a set of 84 RILs resulting from a cross between *S. lycopersicum* and *S. pimpinellifollium* (López-Delacalle et al.,

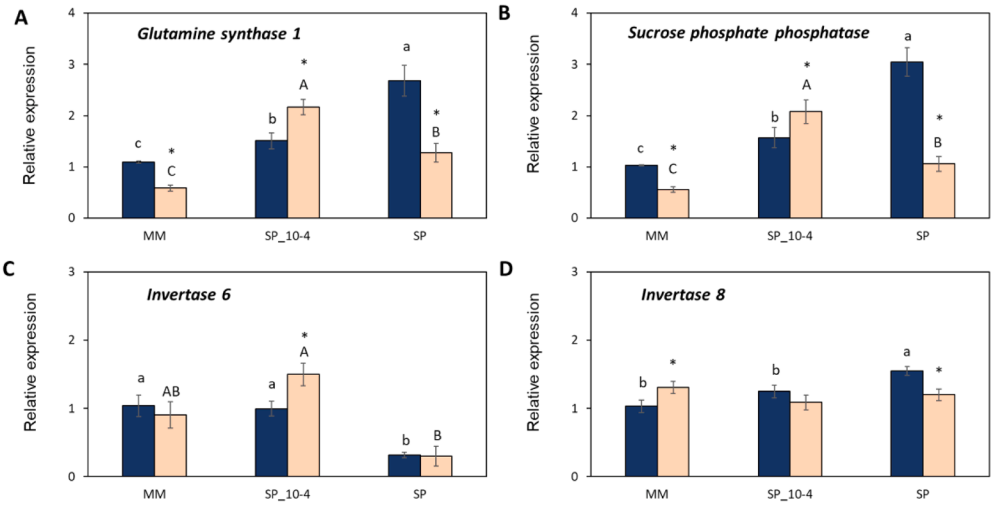

Fig. 6. Relative expression of selected genes contained in the specific region of SP_10-4 under sufficient (8 mM N; blue bars) and limiting (4 mM N; orange bars) N supply conditions. mRNA levels of *glutamine synthetase 1* (A), *sucrose phosphate phosphatase* (B), *invertase 6* (C) and *invertase 8* (D) in leaves of SP_10-4 as well as MM and SP parents. *UBI3, CAC* and *GAPDH* genes used as reference genes. Each value (±SE) is the mean of three different determinations. For each N level, different letters indicate significant differences among genotypes ($P < 0.05$). For each genotype, significant differences ($P < 0.05$) by effect of N supply are indicated by an asterisk.

2020) under control conditions. Furthermore, significant differences were also observed in the growth, photosynthetic and yield traits of *S. penellii* ILs (Halperin et al., 2017; Kang et al., 2021) and in the yield of *S. habrochaites* ILs (Hanson et al., 2007). Together, these results confirm the good potential of the wild alleles to improve the quantitative characters in tomato introgression lines.

Significant phenotypic variation in growth and yield-related parameters was observed in the IL collection analyzed in this study under both N supply conditions (Figs. 2 and 3). Interestingly, the higher N concentrations observed in several ILs suggests that the introgressed SP chromosome segments were associated with the uptake, assimilation and use of N. López-Delacalle et al. (2020) characterized two recombinant lines from a cross between *S. pimpinellifolium* and *S. lycopersicum* showing a more efficient N metabolism related to higher expression levels of N assimilation genes, such as *nitrate reductase* (*NR*), *nitrite reductase* (*NiR*) or *glutamate dehydrogenase* (*GDH*). These genes have proven to be promising targets for the improvement of NUE in crops (Beatty and Good, 2018). However, further studies are required to identify candidate genes in the ILs selected in our study, associated with the increased N contents and improved photosynthetic capacity.

The screening of germplasm during the vegetative stage for high nutrient efficiency or yield, based on shoot biomass, as well as on uptake and utilization efficiencies, would speed up the characterization and phenotyping studies of germplasm collections (Liao et al., 2008). Nevertheless, we reported no correlations between plant biomass in the vegetative stage and fruit biomass in the reproductive stage. These data suggest that the physiological processes involved in the C/N metabolism and growth among the ILs might be differentially affected by the developmental stage. In fact, there is a significant rise in N demand in tomato crops when switching from the vegetative to the reproductive stage and only 25% of the total N demand is absorbed during the vegetative stage (Tapia and Gutierrez, 1997). Accordingly, differences in physiological traits, such as photosynthetic capacity, WUE or stress tolerances, have been reported between the vegetative and reproductive growth stages in tomato accessions (Vicente et al., 2011; Liu et al., 2019; Conti et al., 2021). Tomato germplasm characterizations for the responses of yield and quality traits to N availability have been conducted during the whole life cycle of the plants (Rosa-Martínez et al., 2021; Zhang et al., 2021). However, these phenotypic measurements require specialized personnel and extensive investments in field or greenhouse facilities. Although some studies into tomato identified cultivars or accessions with contrasting NUE characters during the vegetative stage (Abenavoli et al., 2016; Lupini et al., 2017; Kołton et al., 2020), to the best of our knowledge the performance of these genotypes has not been confirmed at the reproductive stage. Our results confirm the necessity of addressing whole life cycle assays for the selection of tomato genotypes with improved NUE.

Nitrogen is the most limiting nutrient for tomato growth. Suboptimal levels of nitrogen may reduce tomato vegetative growth, particularly that of the leaves, and can also have a negative effect on production in terms of the number of fruits and fruit size and may also impact fruit quality, color and taste (Sainju et al., 2003; Renau-Morata et al., 2021). We observed a high variability among ILs in the responses of the different measured biomass and physiological parameters to the drop in N fertilization (Figs. 2, 3 and 4). Of these, SP_10-4 showed no reduction in either the N content or the vegetative biomass; more importantly, there was no reduction in yield when grown under limiting N supply conditions.

The analysis of the specific introgressed region in SP_10-4 permitted the identification of four candidate genes (*glutamine synthetase 1, GS1; invertase 6, INV6; invertase 8, INV8* and *sucrose phosphate phosphatase; SPP*) associated with the greater photosynthetic capacity and biomass production under N deficiency conditions. *Glutamine synthetase 1* encodes for the cytosolic glutamine synthetase isozyme (EC 6.3.1.2) that has been described as involved in N mobilization in tomato under different stress conditions, including pathogen attack (Pageau et al., 2006) and N deficiency (Renau-Morata et al., 2020). Several QTLs for grain yield and grain N content colocalize with *GS1* genes in cereals, and the overexpression of specific *GS1* isogenes resulted in increased growth and NUE in different plant species (Thomsen et al., 2014). Sucrose phosphate phosphatase (SPP) and sucrose phosphate synthase (SPS) catalyze the two-step process leading to sucrose synthesis (Maloney et al., 2015). The repression of *SPP* strongly impairs growth by inhibiting photosynthesis and carbohydrate partitioning (Chen et al., 2005). Finally, invertases catalyze the irreversible hydrolysis of sucrose to free hexoses. Invertase activity has been related to sink strength in many species (Li et al., 2012), but also to the tight regulation of carbon metabolism in leaves (Kingston-Smith et al., 1999).

Phenotypic differences between species developed during the domestication process can be caused by changes in the protein-coding sequences or by changes in the expression patterns of specific related genes (Olsen and Wendel, 2013). In this study we identified non-synonymous mutations in the coding sequences of *GS1, INV6* and *INV8* genes in SP. These allelic variants introgressed in the tomato genome might be related to the improved performance under N limitation. Moreover, changes in the promoter sequences of all four candidate genes were also observed, suggesting that in addition, cis-regulatory variants might be incorporated in the SP_10-4. Notably, the RT-qPCR analysis revealed a drop in mRNA levels of *GS1* and *SPP* in both MM and SP parents in response to N limitation. However, the

expression of these genes in SP_10-4. We hypothesized that the reported changes in the promoter regions might alter the recognition by trans regulatory factors and therefore repress the expression responses to low N. The higher *GS1* and *SPP* expression could be associated with the maintenance of the flux of sugars and amino acids to support fruit growth in SP_10-4 under limited N supply conditions. Nonetheless, it can not be discarded that the allelic variants of *INV6* and *INV8* may also have a significant contribution. Further functional characterization of the candidate genes should be addressed to confirm the relationship of the identified genes with the observed phenotype.

4.3. Identification of genomic regions that have an impact on fruit quality traits in response to N supply

Regarding the impact of introgressions on fruit composition, the only effect observed was the reduction in sugar accumulation and free amino acid content in some ILs. The lack of any positive effects on sugar accumulation was unexpected, as during the initial evaluation of these *S. pimpinellifolium* ILs performed by Barrantes et al. (2016), six consistent QTLs were identified for increased SSC. Other studies have also used the same SP accession to develop RILs, which were screened for QTL effects (Capel et al., 2015). As a result, seven additive QTLs for SSC, two for glucose and one for fructose were also identified. However, once more, these effects were not detected in the present study. The variation in environmental conditions, which can have a profound impact on SSC contents, may account for the differences observed among studies. Under conditions favoring SSC accumulation these QTL effects may be diluted, as both Capel et al. (2015) and Barrantes et al. (2016) reported much lower SSC values than those reached in the present study.

The sugar and acid profile of tomato remained unaltered with suboptimal N fertilization in several ILs. These results basically confirm that tomato fruits maintained C homeostasis under suboptimal N fertilization conditions, showing stable levels of sugars and citric acid accumulation (Renau-Morata et al., 2021). In that study, a substantial reduction (56%) in fruit yield probably helped to maintain that equilibrium. However, in the case of several of the ILs tested here, the sugar and acid profiles remained constant despite maintaining a similar productivity (e.g. SP_10-4), suggesting the existence of different mechanisms.

On the other hand, prominent amino acids were significantly reduced under suboptimal N fertilization conditions in MM and most of the ILs. The effect of N availability on the amino acid accumulation in the fruits is well known. Urbanczyk-Wochniak and Fernie (2005) suggested that aspartic contents would be the most affected under N starvation conditions, followed by glutamic acid, while the GABA contents would be the most stable. However, in the present study, the GABA, glutamine, and glutamic acid contents were more affected than the aspartic acid. In this context, it was highly unexpected the capability of lines, such as SP_1-1, to maintain stable levels of amino acids under suboptimal N conditions and the great accumulation of SP_1-4 under either condition. Increased levels of these amino acids in fruits are becoming an important target in breeding programs. Tomato is one of the main dietary sources of GABA, with valuable hypotensive properties (Nonaka et al., 2017) and neuroprotective and anticancer effects (Ngo and Vo, 2019). In the case of glutamic and aspartic acids, there is an interest in extracting umami-flavor amino acids from tomato paste by-products (Zhang et al., 2015). Furthermore, in specific contexts, glutamic acid can play a positive role in tomato flavor acceptability (Villena et al., 2023).

Few studies have identified the QTLs affecting amino acid concentration in wild species introgressions. Of these, glutamic acid, aspartic acid, glutamine and GABA represent 80% of total free amino acids in tomato (Yilmaz, 2001). Fulton et al. (2002), studying advanced backcross populations from *Solanum habrochaites* Knaap & Spooner, *Solanum peruvianum* L., *Solanum neorickii* Spooner, Anderson & Jansen and *S. pimpinellifolium* (LA1589) under sufficient N conditions, identified several QTLs associated with glutamic acid content, with those with the major effects on chromosome 1, 3 and 8. Furthermore, several QTLs affecting amino acid composition have also been identified in *S. pennellii* introgression lines. Of these, however, SP_1-2 stood out for its high content in both glutamic and aspartic acids (Schauer et al., 2006). Later, this effect was associated with the *SlCAT9* gene coding a tonoplast glutamic-aspartic/GABA exchanger, as its overexpression leads to an increased accumulation of these amino acids (Snowden et al., 2015). It seems then that the performance of SP_1-4 might be justified by an alternative QTL, though it should be checked in alternative environments.

5. Conclusions

The present study addressed the analysis of the *Solanum pimpinellifolium* genetic diversity to improve the response of cultivated tomato to a limitation in N fertilization compatible with sustainable production. We identified specific regions, and proposed candidate genes, in the genome of the wild relative that conferred improved biomass, fruit yield or fruit quality traits to the cultivated tomato under low N inputs. Thus, for future tomato breeding under low N fertilization conditions, some of the current ILs could contribute to an understanding of the genetic basis of the plant response to N limitation and also to the development of new cultivars with enhanced NUE.

Supplemental material

Fig. S1. Positions of the introgressions on the tomato map. Genetic distances (cM) are shown on the left of the chromosome drawings, and physical distances (Mb), according to the tomato genome version SL2.40, on the right (Barrantes et al., 2016). The specific fragment of SP_10.4 compared to SP_10.5 is marked in red.

Fig. S2. Harvest index (HI) in an introgression line (IL) collection of *S. pimpinellifolium* (SP) in tomato (MM) grown under sufficient (8 mM N; blue bars) and limiting (4 mM N; orange bars) N supply conditions during the vegetative stage of growth. Values are mean (±SE) of 6 determinations in different plants. Blue and orange horizontal lines indicate the value for MM in each parameter in 8 and 4 mM N supply conditions, respectively.

Fig. S3. Heatmap of correlations among growth and yield-related parameters evaluated in the introgression line collection of *S. pimpinellifolium* (SP) in tomato (MM) grown under sufficient (8 mM N; below the diagonal) and limiting (4 mM N; above the diagonal) N supply conditions. Parameters labeled in green: vegetative growth stage (Experiment 2019). Parameters labeled in orange: reproductive stage of growth (Experiment 2020). Only significant correlations at $P < 0.05$ according to Pearson test are shown. DW: dry weight; [N]: nitrogen concentration; [C]: carbon concentration; Total N: total nitrogen content; Total C: total carbon content.

Fig. S4. PCA loading plot and score plot for the percentage decrease (PD) in biomass and N use parameters between limiting and sufficient N supply conditions for the IL collection and the parents (Experiment 2020). The first and second components were displayed. Red letters: MM parent; Green letters: ILs. Font size is proportional to fruit size. Acronyms used of the PD between limiting and sufficient N supply were: R[N]Fruit: N concentration in fruits at reproductive stage; R[N]Veg: N concentration in shoots and roots at reproductive stage; R Yield DW: dry matter in fruit at reproductive stage; R Shoot Root DW: dry matter in shoots and roots at reproductive stage; V Root DW: root dry matter at vegetative stage; and V Shoot DW: shoot dry matter at vegetative stage.

Fig. S5. Biomass (vegetative and yield) production of the SP_1-4, SP_3-1 and SP_10-4 plants in greenhouse conditions under sufficient (8 mM N; blue) and limiting (4 mM N; orange) N supply conditions. Experiment (2022) conducted for the confirmation of the previously selected ILs. MM plants served as controls. Each value is the mean (±SE) of determinations in 6 different plants. For each nitrogen level, different letters indicate significant differences ($P < 0.05$). For each genotype, an

asterisk indicates significant differences by the effect of the N supply ($P < 0.05$).

Fig. S6. Principal component analysis biplot of fruit composition from plants of SP_1-4, SP_3-1 and SP_10-4 grown under sufficient (8 mM N; darker color) and limiting (4 mM N; lighter color) N supply conditions (Experiment 2022). The first and second components were displayed. Red circle: MM parent; Green circles: ILs. Dot sizes are proportional to fruit weight.

Fig. S7. Location of the selected genes in the specific region of SP_10-4 introgression line. A) Specific fragment of SP_10-4 compared to SP_10-5. B) Position and exon/intron structure of the three selected genes: C) *invertase 6*, D) *invertase 8* and E) *glutamine synthetase 1*. Data obtained from publicly available resources (http://solgenomics.net).

Fig. S8. Alignments of the sequences of the (A) promoter and (B) coding regions of the candidate genes in the genomes of *S. pimpinellifolium* To-937 and *S. lycopersicum* cv Moneymaker (Gayssant et al., 2022) and the reference tomato (Heinz; www.solgenomics.com). Tomato genes: *sucrose phosphate synthase* (*SPP*, Solyc10g081660.2.1), invertase 6 (INV6, Solyc10g083290.4.1), invertase 8 (INV8, Solyc10g083300.2.1) and glutamine synthetase 1 (GS1, Solyc10g083550.1.1). Non-synonymous changes, deletions and insertions in the coding region were labeled in red.

Table S1. Primers used in RT-qPCR analyses.

Table S2. Mean values of biomass and C/N contents at the vegetative (75 day-old-plants; Experiment 2019) and reproductive (210 day-old-plants; Experiment 2020) stages of growth of the whole introgression line collection (IL) and both parents (MM: *S. lycopersicum* cv Moneymaker and SP: *S. pimpinellifolium* To-937).

Table S3. Mean fruit composition (mg g^{-1} fresh weight) of the whole introgression line collection (IL) and both parents (MM: *S. lycopersicum* cv Moneymaker and SP: *S. pimpinellifolium* To-937).

Table S4. Fruit composition (mg g^{-1} fresh weight) in an introgression line collection of *S. pimpinellifolium* (SP) in tomato (MM) grown under limited (4 mM N) and sufficient (8 mM N) N supply conditions.

Table S5. Carbon and nitrogen metabolism-related genes in the introgressed region of IL10–4 (SL4.0ch10:61800664…62590355). Number of changes in the promotor region (5-UTR ≈ 1 kb) and non-synonymous mutations in the selected genes of the parent S. pimpinellifolium To-937 when compared to the tomato reference genome (ITAG4.0; https://solgenomics.net). Deletions (D no), insertions (I no) and total number of nucleotide changes (no / region length) in To-937 were indicated in the promotor and coding region analysis.

CRediT authorship contribution statement

Begoña Renau-Morata: Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Jaime Cebolla-Cornejo:** Investigation, Formal analysis, Writing – review & editing. **Laura Carrillo:** Investigation. **Daniel Gil-Villar:** Investigation. **Raúl Martí:** Investigation. **José María Jiménez-Gómez:** Formal analysis. **Antonio Granell:** Funding acquisition, Writing – review & editing. **Antonio José Monforte:** Conceptualization, Formal analysis, Writing – review & editing. **Joaquín Medina:** Conceptualization, Funding acquisition, Writing – review & editing. **Rosa Victoria Molina:** Conceptualization, Investigation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing. **Sergio G. Nebauer:** Conceptualization, Investigation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Ministerio de Ciencia e Innovación (MCIN) and the Agencia Estatal de Investigación (grants RTA2015-00014-c02-00 to SGN and JM, PID2020-114165RR-C21 to JM, and PID2022-136541OB-I00 to SGN), the Agroalnext program (MCIN with funding of NextGenEU-PRTR-C17.I1 Generalitat Valenciana AGROALNEXT/2022/056 to SGN), Vicerrectorado de Investigación de la Universitat Politècnica de València (PAID-11-21 to SGN; PAID-10-20 and PAID-PD-22 to RM), Ministerio de Ciencia e Innovación (TED2021-129296B-I00 to SGN) and European Commission H2020 research and innovation program through HARNESSTOM grant agreement no. 101000716 (to AG) and RoxyCOST CA18210 for networking activities (to AG). We also want to acknowledge the "Severo Ochoa Program for Centers of Excellence in R&D" (CEX2020-000999-S) supported by MCIN/AEI/10.13039/501100011033. We thank Mike Bennett for revising the English language and Javier Forment from the IBMCP Bioinformatics service for support on genomic analyses.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2023.112497.

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