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Soil-to-fruit nitrogen flux mediates the onset of fruit-nitrogen remobilization and color change in citrus

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ABSTRACT

In non-climacteric fruit tree species, color change is primarily controlled by the interplay between environmental conditions, nutritional factors (nitrogen and sugars) and hormones, mainly abscisic acid and gibberellins (GA), through a complex mechanism which is not completely understood. Nitrogen has a strong impact on color change, influenced by environmental changes, either locally or at the whole tree level. We use *Citrus* trees, as a non-climacteric model species, to understand the long-distance nitrogen signaling mechanism delaying the chloroplast-to-chromoplast transition at the molecular level. It is unknown whether nitrogen regulates the branch point of geranylgeranyl diphosphate (GGPP), the shared precursor for gibberellin, chlorophyll and carotenoid synthesis. We used ^{15}N to trace the root-to-fruit nitrogen flux in trees grown under soils differing in nitrogen content and temperature, and treated with NH_4NO_3 and GA. Key genes involved in nitrogen signaling, transport and metabolism, and those from the GGPP branch point were analyzed in the fruit. Results explain how soil temperature modifies ^{15}N transport to play a key role in signaling citrus color change, and show that fruit-nitrogen remobilization is required for triggering degreening. Nitrogen content in the fruit modulates the onset of glutamate deamination, asparagine synthesis, nitrite assimilation and GA_1 depletion. Expression of the nitrate transporter NRT1.2, glutamate dehydrogenase and asparagine synthetase genes was high right before the start of degreening, together with NH_4^+ concentration. Nitrogen delayed carotenoid synthesis (phytoene synthase gene expression) without modifying gibberellin synthesis (*ent*-kaurene synthase and oxidase) at the chloroplast level.

1. Introduction

Nitrogen (N) is an essential nutrient for plant development and a key signal molecule regulating metabolic pathways (O'Brien et al., 2016). Fruit trees demand high amounts of N to produce high yields; for instance, 300 kg N ha^{-1} y^{-1} has been reported to be necessary for adult orange (*Citrus sinensis* L.) trees (Quiñones et al., 2003), contrasting with the 150 kg N ha^{-1} y^{-1} for lowland rice (Fageria and Baligar, 2005). But in citrus, applying excess amount of N-fertilizers can reduce fruit quality, and have a damaging impact on the environment. Thus, understanding the effects of N on citrus physiology is crucial to improve N use efficiency in the context of sustainable agriculture.

A key developmental process related with N is fruit ripening, particularly fruit color change. However, the role of the N soil-plant system in citrus fruit ripening remains unclear. Citrus are non-climacteric species in which ethylene plays a secondary role (Alós et al., 2014), and ripening is regulated by a complex network of environmental (light and temperature), nutritional (sugars and nitrogen) and hormonal (gibberellins, ethylene and abscisic acid) factors (Alquézar et al., 2008; Rodrigo et al., 2013).

In citrus grown under Mediterranean climate conditions, the chloroplast-to-chromoplast conversion is completed during autumn and winter, but in Tropical climates, chlorophyll content in the peel does not completely disappear, and the fruit remains practically green at harvest (Reuther and Rios-Castaño, 1969). This climate effect has been explained mainly through the role of temperature. Citrus fruit

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degreening is promoted by low ambient temperatures, below 15 ºC. Particularly important is the low soil temperature (Young and Erickson, 1961) because when temperatures are below 23 ºC citrus root activity is significantly reduced (Bryla et al., 2001), and when they are maintained at 20–23ºC color change is triggered (Mesejo et al., 2012).

Sucrose supplementation *in vitro* or *in vivo* promotes degreening (Huff, 1984, 1983; Iglesias et al., 2001), and sugar concentration in the peel correlates positively with color change (Gambetta et al., 2012; Iglesias et al., 2001). On the contrary, nitrogen treatments *in vitro* block the sucrose effect; overfertilized orchards delay fruit degreening by reducing chlorophyll depletion and carotenoid synthesis (Alós et al., 2006), and nitrogen concentration in the peel negatively correlates with degreening (Gambetta et al., 2012; Iglesias et al., 2001). Among plant hormones, gibberellins (GAs) play a major role in citrus ripening, delaying chlorophyll degradation, senescence, and modifying carotenoid contents and composition (Alós et al., 2006; Gambetta et al., 2014). Moreover, the endogenous content of GA_1 and GA_4 is progressively reduced in the citrus peel during chlorophyll degradation (Gambetta et al., 2012).

The prevailing hypothesis involving environmental and nutritional factors in the control of citrus color change is based on *in vitro* studies with pericarp segments (Huff, 1983): low winter temperatures may induce a decline in nitrogen uptake whereas sugar concentrations in citrus peel continue to increase, both effects contributing to the promotion of color change. However, a cause-effect experiment using trees is needed to test this hypothesis. In citrus grown in the Mediterranean climate, nitrogen uptake is minimum during winter, increases during spring due to higher temperatures and tree demand, peaks in early summer and decreases towards the end of summer and in the autumn (Quiñones et al., 2003). Total nitrogen and proteinaceous fraction concentrations in the fruit diminish with growth and degreening, due to fruit enlargement and protein hydrolysis, whereas fruits prevented from growing due to experimental treatments (branch defoliation or fruit peduncle girdling) have higher nitrogen concentrations and stay green (Gambetta et al., 2012; Iglesias et al., 2001).

Nitrogen depletion occurs in the chloroplasts, yielding glutamine, asparagine, NO_3^- and NH_4^+, the main source for nitrogen remobilization (Bieker and Zentgraf, 2013; Woo et al., 2019). Besides, during the chloroplast-to-chromoplast transition, the geranylgeranyl pyrophosphate (GGPP) branch-point of the MEP (methyl-D-erythritol 4-phosphate) pathway changes from chlorophyll to carotenoid synthesis (Alquézar et al., 2008), and from gibberellins to abscisic acid (Gambetta et al., 2014, 2012). The role of nitrogen in this pathway remains unknown.

In this study, we hypothesize that nitrogen content modulates the onset of the protein hydrolysis during the fruit senescence, which contributes to nitrogen depletion from the fruit as a requisite for color change. Thus, the reduction in nitrogen transport from the root due to low soil temperature is part of the signal that triggers fruit color change. We used ^{15}N to monitor the root-to-fruit nitrogen flux and its effects on fruit color development at the molecular level, by analyzing the expression of key genes involved in N transport, signaling and metabolism, and in the chloroplast-to-chromoplast transition.

2. Materials and methods

2.1. Plant material and growing conditions

Experiments were carried out using 5-year-old potted trees of Clementine mandarin cvs. Loretina and Clemenules (*C. clementina* Hort. ex Tan), and 10-year-old 'Nadorcott' [(*C. clementina* Hort. ex Tan. x *C. sinensis* (L.)] trees. Trees were grafted on the Carrizo citrange [*C. sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.] rootstock. All these cultivars respond equally to nitrogen and gibberellins and present the same mechanism in terms of ripening. 'Clementine' trees were grown outdoors in 40 l pots filled with sandy loamy soil, pH 6.5, at our experimental orchard (Universitat Politècnica de València, Spain), with drip irrigation and fertilization. 'Nadorcott' trees are planted 6 m × 4 m apart in a farm in Valencia (Spain), with sandy loamy soil (85 % sand) and with drip irrigation and fertilization.

Table 1
Experimental design.

Experiments	Treatments	Expected Phenotype	Method	N
Systemic (root-to-fruit) nitrogen signaling				
I	Clemenules	Control	Potted trees	9
	Loretina	Advance in ripening	Potted trees	9
	Clemenules + soil cooling	Delay in ripening	Potted trees	9
II	Clemenules	Control	Potted trees	9
	Clemenules + soil ^{15}N	Delay in ripening	Potted trees	9
	Clemenules + soil ^{15}N + soil cooling	Similar to Control	Potted trees	9
III	Nadorcott	Control	Field trees	10
	Nadorcott – N fertilization	Advance in ripening	Field trees	10
Local (fruit) nitrogen signaling				
IV	Nadorcott	Control	Field trees	3
	Nadorcott + canopy N	Delay in ripening	Field trees	3
V	Nadorcott	Control	Field trees	3
	Nadorcott + canopy GA	Delay in ripening	Field trees	3

2.2. Experimental design, tree phenotyping, and sampling

A total of five experiments were carried out (Table 1). To study the root-to-fruit long distance nitrogen signaling three experiments were carried out during two consecutive years. The first experiment aimed to determine the relationship between root activity and fruit ripening. A set of 'Clemenules' mandarin potted trees (control) was compared with (1) a set of the early ripening 'Loretina' mandarin, and (2) a set of 'Clemenules' trees with a lowered soil temperature, a treatment which advances ripening. We measured the time-course changes in fruit color development, root growth, and N content in the peel of the fruit.

The second experiment aimed to trace the root-to fruit N flux in overfertilized trees under control and lowered soil temperature conditions. A set of control trees was compared with trees overfertilized with $^{15}NO_3K$ (including a 5 % isotopic enrichment in ^{15}N), to delay color change, and with another set of trees overfertilized and grown in reduced soil temperatures, to compensate the N-overfertilization effect. We measured the time-course changes in fruit growth and color, soil temperature, GA_1 and ^{15}N content in the fruit, and, at harvest time, the ^{15}N partitioning in the whole tree.

In both experiments, to maintain soil temperature below 20 ºC, potted trees were placed in polystyrene containers leaving the trunk and the canopy outside, and frozen plastic ice blocks were periodically put into the containers. We used a data logger (WatchDog 400 Spectrum, USA) for each tree to measure soil temperature every hour at 10 cm in depth. The reduction in soil temperature started when the fruit had reached 70 % of its final size (September), two months before full ripening. All the trees were fertilized at a rate of 150 g N $year^{-1}$·$tree^{-1}$ from March to August. Nitrogen was supplied as potassium nitrate (40 %) and calcium nitrate (60 %). Further, overfertilization in the second experiment started in September, treating with an extra amount of 50 g N $tree^{-1}$ split into 16 applications during two months.

The third experiment was performed in orchard conditions using 'Nadorcott' mandarin trees. A set of ten trees was deprived of N fertilization during two consecutive years, and the time-course changes in N content in the spring flush leaves and fruit ripening were measured and

compared with those of the control trees.

To study the local N-signaling in the fruit ripening process, two more experiments in two consecutive years were designed in orchard conditions using 'Nadorcott' mandarin trees. In the fourth experiment, three trees received five biweekly NH_4NO_3 (1.5 % w/v) spray applications (4–5 l $tree^{-1}$), to the whole canopy, starting at the mature-green fruit stage and ending at the fully ripened stage (75-day period). The fifth experiment had the same design but using GA_3 (25 mg l^{-1}) to delay fruit ripening. In both experiments, the treatment solution contained a non-ionic wetting agent at a concentration of 0.05 % (v/v). We measured the time course changes in fruit color, ammonium concentration in the peel, and the relative gene expression in the peel of key genes regulating the process of N assimilation, remobilization and transport, and those of the three routes derived from the precursor GGPP: GA, carotenoid and chlorophyll synthesis.

During the five experiments, we sampled at least three fruits per tree on each date. The peel was frozen in liquid nitrogen and lyophilized, and stored at − 80 ºC. In the second experiment, we sampled and stored as above bark tissues and leaves of fruiting and vegetative shoots, together with xylem, fibrous and lateral roots from three trees per treatment. In the first and second experiments, a sample of fibrous roots from each tree was not frozen and used to measure the number of new lateral root primordia. We also measured total N, ^{15}N, ammonium and GA_1 concentrations, as well as relative gene expression. Diameter and fruit color were regularly measured on the tree.

2.3. Analytical procedures

Fruit color was evaluated with a colorimeter (Chroma Meter CR-400, Konica Minolta, Japan) in ten fruits per tree. The color coordinate *a* indicates green when < 0 and red when > 0. In citrus, the color coordinate *b* is always > 0 and means yellow. To determine fruit coloration the CI ratio was used (CI = 1000·a / b·L).

The number of new lateral root primordia was measured as previously described (Canellas et al., 2002). Briefly, five roots per tree, about 5 cm in length, were boiled at 75 ºC for 20 min in KOH (0.5 %, w/v) for sterilization. Afterwards, roots were washed in water and stained in hematoxylin staining solution for 14 h in the dark [1:40 dilution of a solution having 1 g hematoxylin, 50 ml acetic acid (45 % w/v) and 0.5 g ferric ammonium sulfate]. Finally, roots were washed in water and distained in lactic acid (80 % w/v) at 75 ºC for 1 min. The number of mitotic sites is visible due to red-brown staining, and the sites were counted using a Nikon binocular zoom stereomicroscope. To measure root growth, three pots per treatment were transparent and used as rhizotron, which served to periodically measure root length.

Proteinaceous–nitrogen (N–prot) and ammonium (NH_4^+) were determined as previously described (Reig et al., 2013). In brief, 500 mg (DW) of powder samples were homogenized in 10 ml of trichloroacetic acid (5 %, w/v), at 4 ºC, during 15 min. We added 30 ml of cold trichloroacetic acid (5 % w/v), which was added to the sample. After storing the sample at 4 ºC (15 min) we filtered it across 90-mm filter paper (Albet, Barcelona, Spain). We rinsed the residue, which contained the proteinaceous fraction, four times with 10 ml of cold trichloroacetic acid (5 % w/v). The filtered solution was made up to 100 ml with MQ water and stored, until NH_4^+ analysis, at 4 ºC. For N–prot analysis, both the solid residue and the filter paper were digested using the micro-Kjeldahl method, with 96 % H_2SO_4 (10 ml), H_2O_2 (10 ml) and 3 g catalyst mixture [K_2SO_4:$CuSO_4$:Se (10:1:0.1)], at 450 ºC for 30 min. Then, the digested sample was distilled with NaOH (40 % w/v), boric acid (2 % w/v) and titrated with HCl (0.1 M), in an Auto Distillation equipment (Foss KjeltecTM 2200, Höganäs, Sweden). We express the results as mg N–prot g^{-1} DW. Finally, ammonium was determined with a flow injector analyzer (FIAstarTM 5000, Foss, Höganäs, Sweden), using MQ water as a carrier. NaOH (0.5 M) and the acid–base indicator solution were reagents 1 and 2, respectively. The filtered solution (40 µl) was injected into the carrier stream, and the reaction color change was measured at 590 and 720 nm. Finally, we express the results as µg NH_4 g^{-1} DW.

Labeled ^{15}N abundance was measured with an Elemental Analyzer (NC 2500 Thermo Finnigan, Bremen, Germany) joined to an isotope ratio mass spectrometer (Delta Plus, Thermo Finnigan). We determined ^{15}N by deducting natural ^{15}N abundance from ^{15}N enrichment of each sample, considering the natural atmospheric ^{15}N abundance (0.3663 atom%). The ^{15}N content was calculated according to $^{15}N(mg) = N\ (\%, w/w) \cdot DW\ (g) \cdot atom\%\ ^{15}N\ excess \times 10^{-1}$. The nitrogen derived from the fertilizer (Ndff) was also calculated with the formula Ndff = [(atom

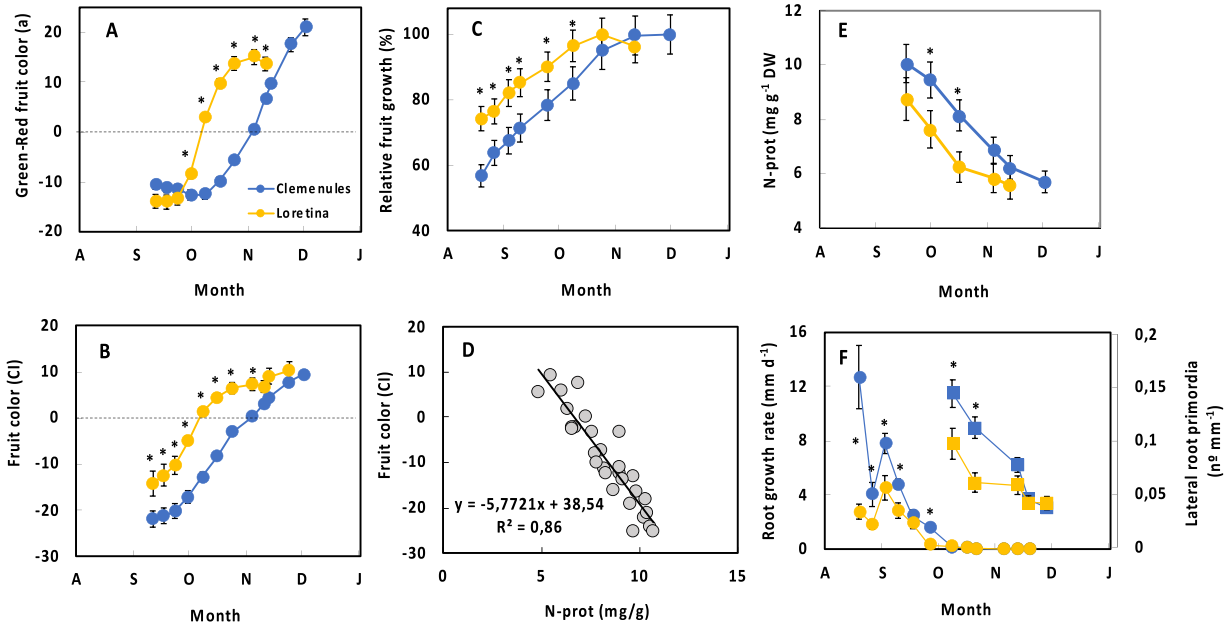

Fig. 1. Time-course changes in fruit color (A and B), fruit growth (C), nitrogen concentration in the peel (E), root growth rate (F, circles) and lateral root differentiation (F, squares) of *Citrus clementina* 'Loretina' (yellow) and 'Clemenules' (blue) potted trees. Correlation between the nitrogen concentration in the peel and the fruit color, using both 'Loretina' and 'Clemenules' data (D). 'a': Hunter-lab coordinate which expresses the green (a<0) and red (a>0) colors; CI= (1000 *a)/(b*L); broken line indicates the fruit color change stage; vertical bars show the standard error of 6 trees; * indicates significant differences ($P < 0.05$).

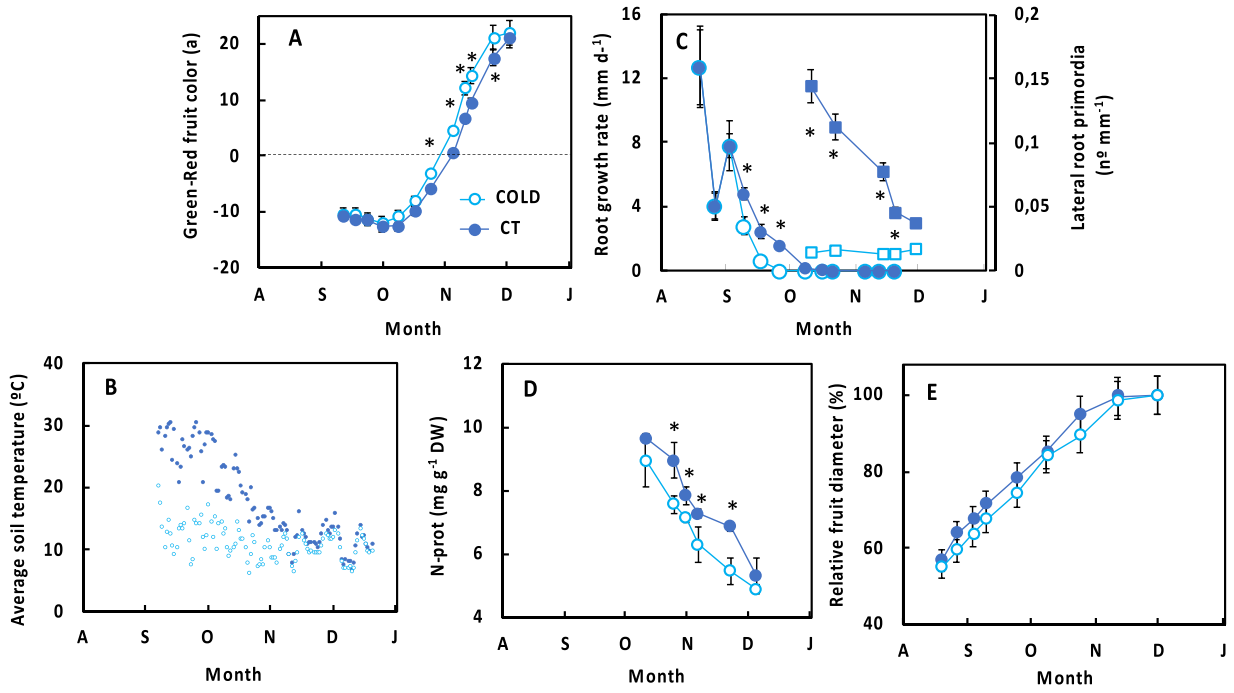

Fig. 2. Time-course changes in fruit color (A), root growth rate (C, circles), lateral root differentiation (C, squares), soil temperature (B), nitrogen concentration in the peel (D) and fruit growth (E) of *Citrus clementina* 'Clemenules' potted trees. Dark blue: control trees; Light blue: low soil temperature; 'a': Hunter-lab coordinate which expresses the green (a<0) and red (a>0) colors; broken line indicates the fruit color change stage; vertical bars show the standard error of 6 trees; * indicates significant differences ($P < 0.05$).

$\%^{15}N_{tissue}$)-(atom$\%^{15}N_{air}$)] / (atom$\%^{15}N_{excess}$) (Quiñones et al., 2003; Martínez-Alcántara et al., 2012).

GA contents in fruit peel were determined by Dr. E. Carrera (IBMCP, Valencia, Spain). An UHPLC-MS was used, with a Q-Exactive spectrometer and an Accucore RP-MS column (2.6 mm, 50 3 2.1 mm; Orbitrap detector; ThermoFisher Scientific) (Camut et al., 2021). In brief, powdered samples were suspended and mixed with the GA internal standards (Olchemim) during 60 min at 4 ºC, in methanol (80 %) and acetic acid (1 %). After storing the extract at − 20 ºC overnight, it was centrifuged to keep and dry the supernatant using a vacuum evaporator. After that, the dry residue was suspended in acetic acid (1 %) and passed through an Oasis HLB column. Finally, the dried eluate was suspended in acetonitrile (5 %)- acetic acid (1 %), and the GAs were separated at 400 ml/min over 14 min by UHPLC, with an acetonitrile gradient of 5–50 % containing acetic acid (0.05 %). Selected ion monitoring (SIM) was used to analyze GA_1 concentrations with the Xcalibur 2.2 SP1 build 48 and TraceFinder programs.

The RNeasy Plant Mini Kit (Qiagen, USA) was used for RNA isolation from frozen tissue, following the manufacturer's instructions. We tested the RNA quality with a Nanodrop ND-1000 spectrophotometer, using the OD260/OD280 ratio, and gel electrophoresis. The QuantiTect® Reverse Transcription Kit (Qiagen, USA) was used to produce cDNA from 1 µg total RNA. And, finally, we carried out the quantitative real-time PCR with the QuantiTect® SYBR® Green PCR Kit on a Rotor Gene Q 5-Plex (Qiagen, USA). The PCR mix contained a 4-fold cDNA dilution (2.5 µl), 0.3 µM primers (1.5 µl), QuantiTect® SYBR Green PCR Master Mix (12.5 µl) (Qiagen, USA), having a final volume of 25 µl. Amplification consisted of a pre-incubation of 15 min at 95ºC, denaturation during 40 cycles of 15 s at 94 ºC, 30 s at 60 ºC for annealing and 30 s at 72 ºC for extension. The Phytozome v13 database (www.phytozome.net) was used to obtain gene sequences. Primers were designed using Primer 3 v4.1.0. software (http://primer3.ut.ee/). The sequences of the primers are specified in Supporting information Table S1.

2.4. Statistical analysis

To determine statistical differences, the experimental data were analyzed by ANOVA and the least significant difference (LSD) test for means separation, with the software Statgraphics Centurion (Statpoint Technologies Inc., USA). To illustrate variations among samples, we calculated the standard errors of means, which were reported in the figures.

3. Results and discussion

3.1. Fruit color correlates with depletion of root-growth and N-transport

Under Mediterranean climatic conditions, Clementine mandarins 'Loretina' and 'Clemenules' naturally ripen during early and mid-autumn, respectively. Thus, during the study the varieties differed significantly in the onset of fruit degreening and carotenoid synthesis. While 'Loretina' fruit started to degreen in late September (Northern Hemisphere), 'Clemenules' did so in mid-October (Fig. 1A). Further, carotenoid biosynthesis was faster in 'Loretina' than in 'Clemenules', as shown by the kinetic of *a* value and the color index (CI) (Fig. 1A, B). Both *a* (green-red) and *b* (yellow) coordinates significantly boosted the CI in 'Loretina' compared to 'Clemenules'. In both genotypes, degreening started when fruits had reached 90 % of their final size (Fig. 1C), indicating the onset of the ripening stage (Agusti et al., 1997).

Fruit color correlated inversely with peel N content (proteinaceous fraction, N-prot) (Fig. 1D) shown by the faster depletion of N-prot content in 'Loretina' compared with 'Clemenules' (Fig. 1E). Fruit degreening also correlated inversely with root growth for both cultivars, as previously described in other species (Reig et al., 2013). In fact, 'Loretina' trees cease root elongation and lateral root primordia appearance (Fig. 1F). In both cultivars, root growth rates became negligible coinciding with the onset of ripening (Fig. 1F). Since rootstock, soil, and climatic conditions were the same for all the trees, the effect on root depletion must be associated to the canopy cultivar. This

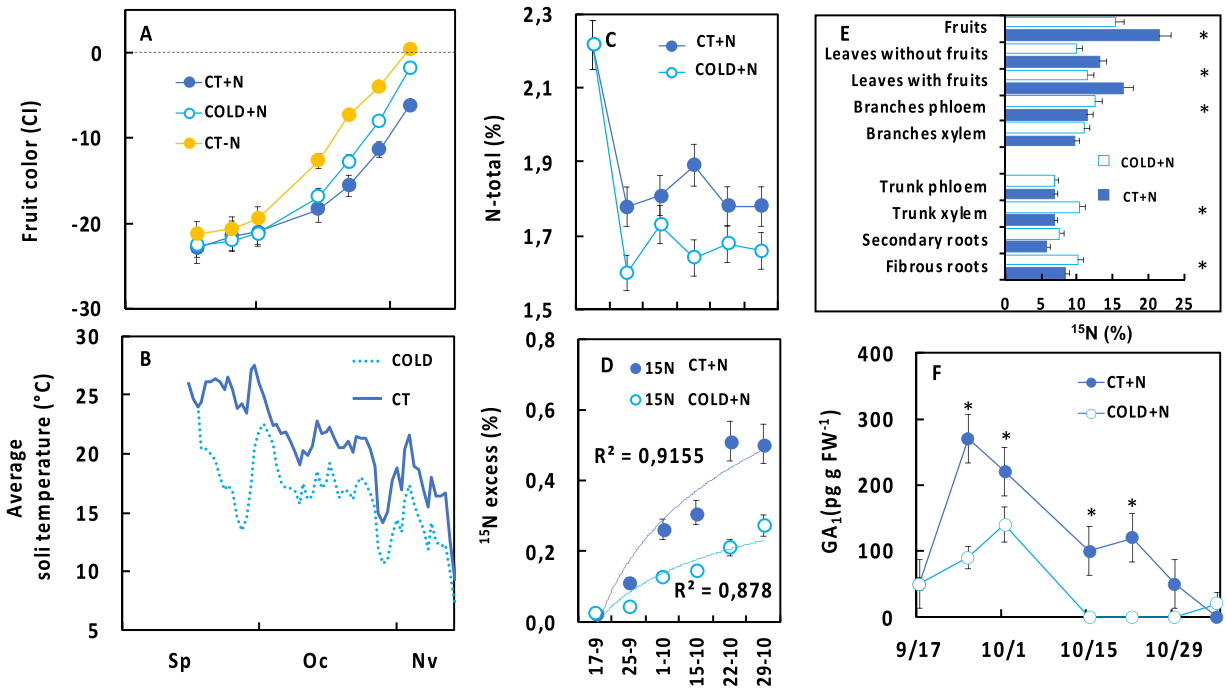

Fig. 3. Time-course changes in fruit color (A), soil temperature (B), total-N (C), excess ^{15}N (D) and GA_1 (F). Tree partitioning of ^{15}Ndff (E). *Citrus clementina* 'Clemenules' potted trees were continuously fertilized with $^{15}NO_3K$ (dark and light blue) in control pots (yellow, dark blue) or pots with low soil temperature (light blue). The soil fertilization treatment started the 15th of September and was split into 16 applications during two months; CI= (1000 *a)/(b*L); broken line indicates the fruit color change stage; vertical bars show the standard error of 6 trees; * indicates significant differences ($P < 0.05$).

effect was explained in terms of sink strength and fruit-root competition for carbohydrates (Reig et al., 2013).

Thus, fruit color development correlates with diminished root growth and depleted nitrogen content in the peel. However, these correlations do not demonstrate causality, i. e. lower root growth does not strictly imply lower nitrogen transport to the fruit. Therefore, to determine the soil-to-fruit nitrogen flux and its effect on fruit color change, new experiments were conducted using ^{15}N and forcing root depletion.

Soil cooling from late summer (September, NH) until harvest advanced peel degreening (Fig. 2A, 2B), and induced root depletion through a full inhibition of root elongation and lateral root primordia differentiation (Fig. 2C). These correlated to a faster reduction in the N-prot content in the fruits (Fig. 2D). Reduced soil temperature did not significantly modify fruit diameter (Fig. 2E), which suggests that the reduction in N-prot content was due not to a dilution effect induced by fruit growth, but to a metabolic effect.

To trace N movement in the soil-plant system, and hence determine the N flux from the root to the fruit, ^{15}N labeled fertilizer was applied to trees growing both in natural or cooled soil. We tested the hypothesis that continuous N supply ($K^{15}NO_3$) to the soil from late summer to the harvest date reaches the fruit delaying degreening, the effect being counteracted by soil cooling. The results showed that peel degreening in the control untreated trees started the first half of October (Fig. 3A), coinciding with the date at which the average soil temperature reached 20–23 ºC (Fig. 3B). Although root uptake is progressively diminished during autumn due to low temperatures (Martínez-Alcántara et al., 2012; Quiñones et al., 2003), continuous $K^{15}NO_3$ supply to the soil led to an increase in ^{15}N enrichment in the fruit (Fig. 3D), significantly delaying fruit degreening by two weeks (Fig. 3A), and N-total depletion (Fig. 3C). An extra reduction in soil temperature (apart from the natural reduction during autumn) was needed to limit the translocation of ^{15}N to the fruit (Fig. 3D), and partially counteract the $K^{15}NO_3$ effect in fruit color (Fig. 3A). Differences in ^{15}N enrichment were not due to differences in tree biomass, because the trees were similar at the beginning of the experiment and they did not growth during the experimental period (autumn).

The results indicate that N uptake and translocation to the fruit was not inhibited, but it was progressively reduced according to the logarithmic tendency observed in ^{15}N excess fruit content (Fig. 3D). The significant reduction in ^{15}N in the fruit due to soil cooling might be an effect of lower ^{15}N uptake by roots, ^{15}N translocation, or both. The results obtained through the ^{15}N partitioning in the plant (Fig. 3E) suggest that it might be a question of lower ^{15}N translocation rather than lower ^{15}N root uptake; in fact, roots and trunk had higher amounts of ^{15}N whereas that of leaves was lower in cooled trees compared to the control ones (Fig. 3E). Therefore, it is concluded that cold soil temperatures reduce N flux from the roots to the fruit, reduce N content in the leaves and fruits, and allow for fruit degreening. Differences in N partitioning between control and cold soil treated trees could be due to a priorization to store it in roots and trunk as a response to cold temperature stress (Millard and Grelet, 2010).

But in citrus trees, not only does the soil provide N to the fruit, it also receives about 65% of total N remobilized from storage organs (old leaves) during spring and early summer, and about 35 % from the soil during summer and early autumn. The lower N availability in the soil, the higher N remobilization from storage organs (Martínez-Alcántara et al., 2012). Thus, to better understand the role of soil N availability in fruit ripening, we studied a set of trees grown in a field with very low nitrogen availability (85% sandy soil conditions with 0.2 % organic matter). The field was left unfertilized with N during two consecutive years in order to ensure that almost all the N content of the fruit came from tree reserves. The results show that unfertilized trees remobilized a great amount of N from the leaves during the spring. The reduction in soil-N availability significantly increased fruit coloration and advanced the onset of fruit degreening by ten days, approximately, compared to the control trees, thus confirming the importance of soil-N availability and translocation to the fruit inhibiting peel ripening (Figure Supplementary 1).

To test whether N flux from the soil is linked to GAs in the fruit, we also measured the contents of bioactive GA_1 in response to ^{15}N

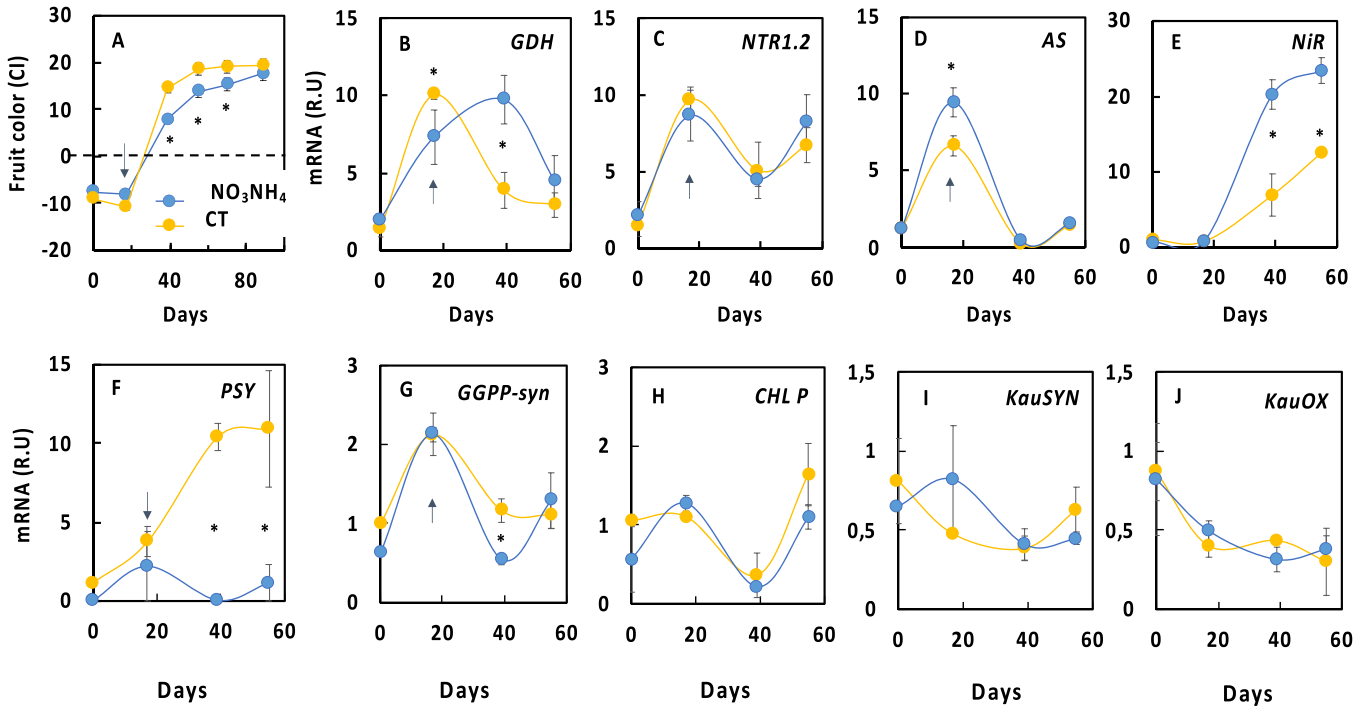

Fig. 4. Time-course changes in color of 'Nadorcott' mandarin fruits (A), and gene expression of *GDH* (B), *NRT1.2* (C), *AS* (D), *NiR* (E), *PSY* (F), *GGPP-syn* (G), *CHL P* (H), KS (I) and KO (J), involved in glutamate deamination (B), nitrogen transport (C, D), ascorbate synthesis (D), nitrite reduction (E), phytoene synthesis (F), GGPP synthesis (G), chlorophyll synthesis (H) and kaurene synthesis and oxidation in the first step of gibberellin synthesis. Trees were treated five consecutive times with NH_4NO_3 (1.5 %) at days 0, 17, 39, 55, and 70; broken line indicates the fruit color change stage; vertical bars show the standard error of 3 trees; * indicates significant differences ($P < 0.05$).

overfertilization. Almost two weeks before the onset of degreening (Sept 25th, CI = - 21, Fig. 3A) the contents of bioactive GA_1 in the fruit peel decreased progressively (Fig. 3F) with increased ^{15}N content (Fig. 3D), suggesting that GA depletion is triggered regardless of N. However, the significant reduction in ^{15}N in the fruit due to soil cooling correlated to the contents of bioactive GA_1 in the peel (Fig. 3F), suggesting a link between N signaling and GA metabolism, during citrus color change, as observed in other plant systems (Camut et al., 2021). In particular, our findings suggest that N mediates GA metabolism delaying GA_1 depletion in the peel, which is consistent with the effect of GA treatments delaying

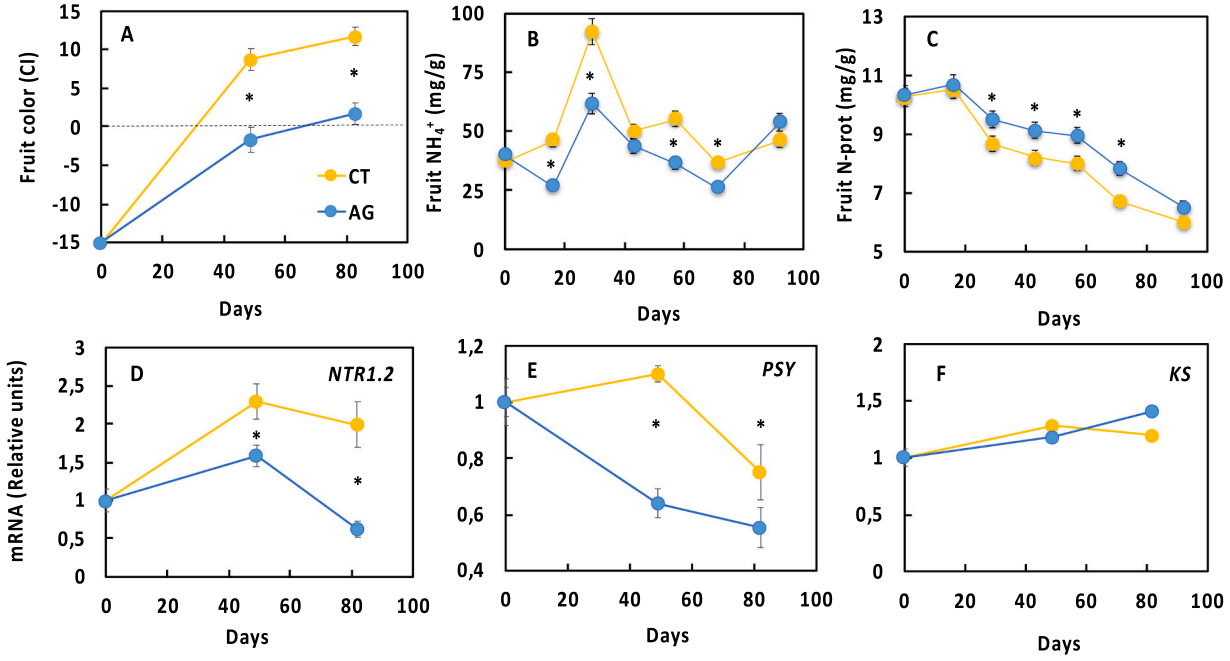

Fig. 5. Time-course changes in fruit color (A), ammonium (B) and N-prot (C) content in the peel, and gene expression of NRT1.2 (D), PSY (E) and KS (F) of 'Nadorcott' mandarin fruits. Genes are involved in nitrogen transport (D), phytoene synthesis (E), and kaurene synthesis in the first step of gibberellin synthesis (F). Trees were treated four times with GA_3 (25 mg l^{-1}); broken line indicates the fruit color change stage; vertical bars show the standard error of 3 trees; * indicates significant differences ($P < 0.05$).

color change (Alós et al., 2006), and agree with previous results, indicating the inverse relationship between endogenous GAs and citrus peel color (Gambetta et al., 2012). Does N modify GA synthesis or catabolism? In the context of low N availability, the contents of bioactive GA_1 mediates the synthesis of red pigments mostly depending on GA catabolism (GA2ox activity) rather than GA synthesis (GA3ox activity) (Zhang et al., 2017). In subsequent experiments, we delayed ripening by 1) maintaining a high N concentration in the peel by continuously treating the canopy with NH_4NO_3 (fourth experiment) and 2) treating with GA_3 without modifying N dose (fifth experiment), to investigate whether peel N remobilization is locally associated with color change and the GGPP branch point (synthesis of GAs at the chloroplast level, carotenoids and chlorophylls).

3.2. *Nitrogen modifies the onset of glutamate deamination and carotenoid synthesis, but not other routes derived from GGPP, during the chloroplast-to-chromoplast conversion*

Given that N remobilization takes place mainly as glutamine, glutamate, asparagine and aspartate (Okumoto and Pilot, 2011; Woo et al., 2019), we studied the expression of the genes codifying the enzyme glutamate dehydrogenase (GDH) and asparagine synthetase (AS). Besides, we studied the N membrane transporters from the NPF family (Léran et al., 2014), specifically, the nitrate transporter NPF4.6/NRT1.2 found to be upregulated in early varieties of the Clementine mandarin, suggesting that it may play a key role in citrus color change (Ríos et al., 2010). NRT1.2 is also involved in N signaling (O'Brien et al., 2016).

Continuous NH_4NO_3 treatment directly to the fruit delayed but did not prevent chloroplast-to-chromoplast conversion (Fig. 4A), as occurred with continuous KNO_3 treatment to the soil (Fig. 3A). GA_3 treatment induced a similar effect on fruit color change, although more effectively (Fig. 5A and (Alós et al., 2006)). The results of *GDH* relative expression levels showed an upregulation (10×) of the gene in control fruits (Fig. 4B), triggering glutamate degradation previous to fruit color change. *GDH* is involved in the deamination of glutamate during protein hydrolysis, which agrees with the NH_4^+ peak detected just at this moment, *i.e.* the onset of peel degreening (Fig. 5B). *GDH* upregulation was delayed significantly in NH_4NO_3 treated fruits, in which *GDH* expression peaked 22 d later, *i.e.*, 39 days after treatment (DAT). At this point, NH_4NO_3 treated fruits still showed a significantly lower color than control fruits. These results suggest that protein catabolism could be postponed due to NH_4NO_3 application and that fruit color change is linked to the onset of protein hydrolysis at a transcriptional level. Our hypothesis is supported by the GA_3 effect delaying N-prot depletion (Fig. 5C) and reducing NH_4^+ concentration in fruit peel (Fig. 5B), probably derived from deamination.

Concomitantly, nitrate transporters should be involved in the translocation of the N-prot catabolites. In fact, *NPF4.6/NRT1.2* expression levels in the control trees peaked together with *GDH*, although the NH_4NO_3 treatment did not significantly modify *NPF4.6/NRT1.2* expression patterns (Fig. 4C). However, in both cases, *NPF4.6/NRT1.2* reached the highest relative expression right before degreening onset, which shows that N mobilization is required. On the other hand, GA_3 reduced *NPF4.6/NRT1.2* expression patterns in green fruits compared with control fruits undergoing color break (Fig. 5D), an effect perhaps related with the lower N-prot depletion (Fig. 5C). *NPF4.6/NRT1.2* was previously found to be downregulated in green citrus fruits compared to those undergoing color break (Ríos et al., 2010), which agrees with our results.

The expression of *AS* also peaked ahead the onset of fruit degreening in control trees, and was slightly higher in the NH_4NO_3 treated fruits (Fig. 4D). *AS* expression is induced in senescing organs (Lin and Wu, 2004), and our results support the hypothesis that asparagine plays a role as a form of N remobilization previous to color change in citrus as well. Beyond N translocation, the application of NH_4NO_3 to the canopy triggered a restart in nitrate assimilation through the upregulation of nitrite reductase (NiR) enzyme (Fig. 4E), not correlated to the delay in fruit color as happens afterwards.

Altogether these results suggest that the delay in deamination of glutamate during protein hydrolysis (GDH activity), N-prot depletion, and also NO_3^- and NH_4^+ remobilization in the chloroplast, might be key factors involved in N metabolism and color change. The excess of N applied is probably evacuated first in the form of asparagine (as *AS* is overexpressed in treated fruits), and thereafter assimilated as a simple form of nitrogen (an increase in the relative expression of *NiR* is achieved later in treated fruits). The N transporter gene (*NPF4.6/NRT1.2*) showed an increase in its expression prior to the onset of color break regardless of the N application. Although other N transporters might be involved in the process of N translocation (O'Brien et al., 2016), results suggest that the upregulation of this N transporter is a constitutive process required for citrus degreening (Ríos et al., 2010), independent to exogenous N. Its role in the nitrogen signaling pathway should not be discarded.

The NH_4NO_3 and GA_3 treatments also modified the expression of phytoene synthase (*PSY*), the enzyme responsible for the synthesis of phytoene, the carotenoids precursor. *PSY* significantly increased (10-fold) its relative expression during color change in control fruits (about 20 days after the onset of degreening), whereas its expression was blocked in NH_4NO_3 and GA_3 treated fruits (Fig. 4F and 5E). These results match with previous studies at a molecular level, in which fruits treated with nitrate or GAs showed low levels of *PSY* expression and a carotenoid profile typical of immature fruits (Alós et al., 2006).

As the starting point of these metabolic pathways in the chloroplast, the enzyme GGPPsyn synthesizes the precursor GGPP, common to carotenoids, chlorophylls and GAs (Lin and Wu, 2004). Relative expression of *GGPPsyn* increased significantly at the onset of degreening in both control and NH_4NO_3 treated fruits (Fig. 4G), previous to the increase in *PSY* gene expression. The expression of geranil-geranil-reductase (*CHLP*), which triggers the synthesis of chlorophylls from the reduction in GGPP was not modified by NH_4NO_3, and was constitutively expressed during the color change process (Fig. 4H). This could explain why the regreening process did not take place, and that the delay in fruit degreening is not attributable to the reactivation of chlorophyll synthesis. On the other hand, pheophorbide *a* -oxygenase (*PaO*) gene expression, which catalyzes chlorophyll breakdown, was delayed by the NH_4NO_3 treatment (data not shown), as reported previously (Alós et al., 2006). And finally, it is worth noting that both kaurene synthase (*KS*) and kaurene oxidase (*KO*) gene expression were progressively reduced throughout the process of chloroplast-to-chromoplast conversion (Fig. 4I, J), inversely to what occurred with *PSY* (Fig. 4F). Although the continuous application of NH_4NO_3 to the fruit delayed peel senescence, the treatment did not modify the first steps in the synthesis of GAs, which are also localized in the chloroplast. Neither *KS* nor *KO* gene expression was significantly modified by NH_4NO_3 (Fig. 4 I, J) and GA_3 (Fig. 5F). However, the depletion of GA_1 was delayed by N (Fig. 3F). In Arabidopsis, overexpression of *KS* greatly increases the amount of GA_{12}, but this has little effect on bioactive GA concentrations (Fleet et al., 2003). Thus, it is possible that nitrogen regulates the final steps of GA biosynthesis and catabolism as it has been observed in Arabidopsis (Zhang et al., 2017).

In accordance with our results, the role of N in fruit peel is crucial for the onset of degreening during the chloroplast-to-chromoplast transition. High N content is linked to a delay in *GDH* activity and N-prot depletion, a reduction in free NH_4^+ concentration and a delay in N transport and remobilization in the form of asparagine. Previous studies show a link between N content and leaf chloroplast ultrastructure (Bondada and Syvertsen, 2003), supporting our results. Thus, N-deficient citrus plants have small chloroplasts, without starch, low in chlorophyll concentration, and they exhibit a loss of membrane integrity and with a pale-yellow color (Bondada and Syvertsen, 2003). Further, N plays a role in the GGPP branch-point of the MEP pathway, but only

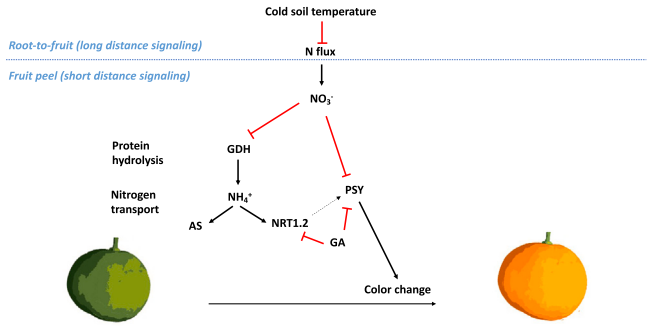

Fig. 6. A model summarizing the regulatory system of nitrogen signaling in citrus fruit ripening. Nitrate in the fruit peel delays protein hydrolysis and senescence by repressing *GDH* induction. When GDH is induced an ammonium peak is produced, which induces nitrogen transporters (*AS*, *NRT1.2*) to contribute to N depletion, and fruit color change is triggered. GAs interfere in *NRT1.2* and *PSY* induction and delay fruit color change. Red and black lines represent repression and promotion, respectively. Dashed arrow represents no direct cause-effect relationship established. N: nitrogen; NO_3^-: nitrate; GDH: glutamate dehydrogenase; NH_4^+: ammonium; AS: asparagine synthetase.

delaying carotenoid synthesis without interfering in GA and chlorophyll synthesis at this developmental stage. Nevertheless, high N content is linked to a delay in GA_1 depletion, which may be considered a consequence of a delay in the process of chromoplast differentiation.

4. Conclusion

In summary, citrus fruit ripening correlates with a depletion of root-to-fruit N translocation, which, in the Mediterranean climate, is linked with a reduction in soil temperature (Fig. 6). The reduction in nitrogen arrival to the fruit signals the onset of *GDH* and *AS* expression in the chloroplast, N-prot depletion, and a reduction in the derived free NH_4^+ concentration and other forms of N, due to its transport and remobilization (*NPF4.6/NRT1.2*), and GA_1 content. Given that low ambient temperature also promotes citrus color change (Carmona et al., 2012), its direct role in N transport and remobilization (*NPF4.6/NRT1.2*) should be taken into account. On the whole, this allows for the onset of chloroplast-to-chromoplast transition and fruit degreening. Further, N delays certain aspects of chromoplast differentiation such as the expression of PSY, but it does not modify the first steps of GA or chlorophyll synthesis at this developmental stage. The application of GA_3 also reduced N-prot depletion, free NH_4^+ concentration, N transport (*NPF4.6/NRT1.2*) and phytoene synthesis. Thus, the reduction in N translocation from the roots to the fruit due to a drop in temperature in autumn plays a decisive role in citrus fruit ripening in the Mediterranean climate, and may explain the lack of degreening in Tropical climates. We propose that root-to-fruit N flux mediates the onset of protein hydrolysis in the chloroplast, which contributes to N and GA_1 depletion from the fruit as a prerequisite for color change.

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CRediT authorship contribution statement

C.M. and M.A. conceived the research and designed the experiments; A.L.O, A.M.F., C.R., G.G., A.M., and B.M.A performed the experiments; C.M., A.L.O, G.G., A.G. and M.A. analyzed and interpreted the data; C.M. and M.A. wrote the manuscript with contributions from all authors.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2022.105088.

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