Short title: Nitrogen-dependent effects of a genetic intervention (50 characters and spaces)

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Nitrogen inputs impact metabolism in vegetative tissues in maize engineered with an endosperm-specific carotenoid pathway[1]

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Abstract (max. 250 words)

An earlier transcriptomic, proteomic and metabolomic analysis between a maize line engineered with an endosperm-specific carotenogenic mini-pathway HC, and its near-isogenic counterpart revealed a concurrent up-regulation of sterol and fatty acid synthesis in the embryo. In this study, we assess, at the metabolic level, whether the endosperm-specific intervention triggered compensatory effects in leaves and roots of HC during vegetative growth. Since plant metabolism is impacted by nutrient supply, we extended our analysis to plants growing under contrasting N regimens. The untargeted metabolomic analysis revealed an increase of organic acids from the tricarboxylic acid (TCA) cycle in HC even under conditions of low N supply. In contrast, soluble sugars, carotenoids, and chlorophyll were decrease under both N levels. Genotype- and N supply-dependent differences are discussed. A model is put forward to explain how the pool of organic acids from the TCA cycle which accumulated during vegetative growth might contribute to the increased metabolite demand of pyruvate and/or acetyl-CoA in the endosperm and embryo. It is likely that the reprogramming of the C primary metabolism we observed in leaves and roots of HC may be attributed at least in part to a transgenerational priming or stress memory induced by the increased demand of metabolic precursors during seed formation in the original generation and was inherited through the germ cells.

Introduction

Carotenoids are natural isoprenoid pigments found in all photosynthetic organisms. They play an important role in light harvesting and photoprotection (Zhu et al., 2013; 2018). In non-photosynthetic tissues such as fruits and flowers, carotenoids confer yellow, orange and red pigmentation to facilitate interactions with animals, for example by attracting pollinators and seed dispersers (Heath et al., 2013). Humans cannot synthesize carotenoids *de novo* and acquire them primarily by eating plant-based foods. Carotenoids are important nutritional factors in humans, with many acting as health-promoting antioxidants. For example, β-carotene has pro-vitamin A activity because it is a precursor of 11-*cis*-retinal, the light-absorbing component of the visual pigment rhodopsin (Rao and Rao, 2007). Carotenoids are also important food additives in the fish and poultry industries because they impart flesh coloring an important organoleptic property (Berman et al., 2015; Breitenbach et al., 2016; Diaz-Gomez et al., 2017).

Many staple cereals do not accumulate carotenoids in sufficient quantities for populations that subsist on a primarily cereal-based diet, leading to pervasive vitamin A deficiency in developing countries (Farré et al., 2010a; 2010b). In some cereals, the carotenoid content has been improved by conventional breeding (Harjes *et al.*, 2008; Yan *et al.*, 2010; Babu *et al.*, 2013; Suwarno *et al.*, 2015) but a more direct approach is metabolic engineering to enhance the production of nutritionally important carotenoids, leading to the development of Golden Rice (*Oryza sativa*) producing β-carotene in the endosperm (Paine et al., 2005; Ye et al., 2000) and

various transgenic rice and maize (*Zea mays*) varieties accumulating higher levels of β-carotene, lutein, zeaxanthin and ketocarotenoids (Bai *et al.*, 2011; Zhu *et al.*, 2013). We previously reported the generation of a maize line based on the South African white maize elite inbred M37W engineered with a carotenogenic mini-pathway (maize *psy1* and *Pantoea ananatis crt*I driven by endosperm-specific promoters) to enhance overall carotenoid production in the kernels (Zhu et al., 2008). The total carotenoid content of this high-carotenoid (HC) maize line increased 140-fold compared to the parental line M37W and the kernels accumulated large amounts of β-carotene (Zhu et al., 2008).

Agronomic practices affect physiological and metabolic traits in crops, including cereals. Several inbred maize lines developed by conventional breeding produce high grain yields and accumulate higher amounts of β-carotene (Harjes et al., 2008; Yan et al., 2010; Babu et al., 2013; Suwarno et al., 2015) and some of these varieties have been grown in several countries in Africa since 2013 (Garg *et al.*, 2018). However, the breeding and evaluation of these hybrids generally takes place under optimal agronomic conditions, including the application of recommended doses of nitrogen (N) fertilizer, which contrasts with the N-limited farming systems typically encountered by low-income farmers in Africa (Morris et al., 2007). A recent field evaluation of 30 high-yielding maize hybrids bred to produce higher quantities of β-carotene showed that N starvation reduced both the yield and the provitamin A content (Manjeru et al., 2019), whereas recommended doses of N fertilizer boosted the provitamin A content in these lines and in 55 additional hybrids (Ortiz-Covarrubias et al., 2019).

We have previously shown that the agronomic performance of HC maize and M37W is similar in the greenhouse and in the field under two different N regimens (Zanga et al., 2016). We found little difference in grain yield between the varieties, and the total carotenoid levels were similar in the greenhouse (96.8 µg g^{-1} dry weight (DW) of seed) and in the field (88.7 µg g^{-1} DW) under both N regimes, although the greenhouse plants accumulated 10% β-carotene as a proportion of total kernel carotenoids compared to 7% in the field (Zanga et al., 2016). In genetic terms, HC and M37W maize differ solely in the endosperm-specific expression of the *Zmpsy1* and *Pacrt*I transgenes, but a comprehensive transcriptomic, proteomic and metabolomic analysis of the endosperm and embryo revealed extensive differences in carbohydrate, fatty acid and sterol biosynthesis in the kernels (Decourcelle et al., 2015). This was attributed to a competition for resources between the introduced mini-pathway and endogenous pathways. The accumulation of carotenoids, fatty acids and sterols in the HC maize imposed a higher demand on the limited pool of precursors and intermediates, reducing the supply of fructose and glucose. The study focused on effects in the kernels as the site of transgene expression but did not investigate whether there was an additional metabolic impact in vegetative tissues caused by sink–source relationships (Smith et al., 2018).

We used an untargeted metabolomics approach to determine whether the engineering of endosperm carotenoid biosynthesis affected metabolism in leaves and roots of HC. More specifically, our hypothesis was that boosting carotenoid biosynthesis in the endosperm would affect primary carbon metabolism in vegetative tissues and that the tricarboxylic acid (TCA) cycle would need to adapt in order to meet the increased demand for products of the glycolytic pathway in the kernels. Nutrient availability affects many physiological and molecular responses of plants, including core metabolism (Amiour et al., 2012; Schluter et al., 2012; Li et al., 2016). We therefore compared HC and M37W plants grown under different N supply regimes to characterize the effect on primary metabolism in the roots and leaves, and to determine whether such regimes had differing effects in the two genotypes.

Materials and methods

Plant material

The M37W and HC varieties of maize (Zhu *et al.*, 2008) were grown in the greenhouse with a 10-h photoperiod (28/20 °C day/night temperature) and 60–90% relative humidity for 3 months (late October to January). Five plants of each genotype were grown individually in 20-L pots for each N treatment regime, making 20 plants in total. Four leaves were harvested from plants with six fully-expanded leaves. The leaf tissue was wrapped in aluminum foil and immediately placed in a Gamma 2-16 LSC plus vacuum drier (CHRIST, Osterode am Harz, Germany) for 48 h. Roots were rinsed with distilled water, dried with paper towels, wrapped in aluminum foil, and dried under vacuum as above. Dried tissue was stored at –20 °C.

N treatments

The plants were grown in substrates containing high or low levels of N, hereafter abbreviated to N+ and N-, respectively. The N+ substrate was commercial sphagnum peat (Klasmann-Deilmann, Geeste, Germany), whereas the N- substrate was a mixture of sand (B-Biosca, Lleida, Spain) and peat (as above) in a 3:1 (v/v) ratio. Soil analysis performed by Eurofins Agroambiental (Sidamon, Spain) showed that the N+ and N- substrates contained 758.5 and 24 mg/kg of NO_3, respectively. Other nutrients were maintained at similar levels in both substrates by adding NPK (0-17-19) liquid fertilizer with micronutrients (Biovert, Corbins, Spain) to plants under the N- regime 15 and 30 days after sowing, and DuraGREEN Sprint NPK (20-5-10) + Ca Mg S (3-2-14) solid fertilizer (Fertinagro Nutrientes, Teruel, Spain) to plants under the N+ regime 7 days after sowing. All plants were watered daily.

Extraction of polar and non-polar metabolites from leaves and roots

Dried leaves and roots were ground to fine powder using a TissueRuptor (Qiagen, Venlo, Netherlands) and 10 mg of the dried powder was extracted in methanol/water according to Bligh and Dyer (1959) with modifications. Briefly, 400 µl of methanol and 400 µl of water were added to the dried material in a microfuge

tube and mixed by shaking for 1 h in the dark. We then added 800 µl of chloroform, mixed the contents by vortexing and separated the phases by centrifugation at 13,000 × g for 5 min at room temperature. We removed 10 µl of the epiphase (containing polar metabolites) for gas chromatography mass spectrometry (GC-MS) analysis and the organic layer (hypophase, containing carotenoids and chlorophylls) was set aside for ultra-high-performance liquid chromatography (UPLC) analysis. A separate set of 10-mg samples was saponified with 1 ml 10% (w/v) NaOH for 1 h in a bath sonicator and centrifuged as above to recover the lipid pellet. This was extracted as previously described (Bligh and Dyer, 1959) and 400 µl of the organic phase was removed for GC-MS analysis of fatty acids, sterols, tocopherols and other isoprenoids.

UPLC analysis

The carotenoid extracts were dried under vacuum, redissolved in 50 µl ethyl acetate and centrifuged at 13,000 × g for 10 min at room temperature. We then injected 3-µl aliquots into an Acquity UPLC system (Waters, Milford, Massachusetts, USA) fitted with an ethylene bridged hybrid (BEH C18) column (2.1 × 100 mm, 1.7 µm) with a BEH C18 VanGuard pre-column (2.1 × 50 mm, 1.7 µm) as described by Nogueira et al. (2013). The mobile phase was a mixture of solvent A (50/50 methanol/water) and solvent B (75:25 acetonitrile/ethyl acetate). All components were HPLC grade and were passed through a 0.2-µm filter before use. The mobile phase was held at 30:70 A:B for 30 s before increasing to 0.1:99.9 A:B for 5.5 min and returning to 30:70 A:B for the last 2 min. The column temperature was maintained at 30 °C and the sample temperature at 8 °C. Continuous online scanning across the UV/visible range (250–600 nm) was carried out using an extended wavelength photodiode array (PDA) detector (Waters). Carotenoids were quantified from the dose–response curves. The chromatographic separation, detection, and quantification of carotenoids, tocopherols, and chlorophylls are described in detail elswhere (Fraser et al., 2000).

GC-MS analysis

The 10-µl polar extracts and 400-µl saponified extracts were transferred to separate glass vials and spiked with the corresponding internal standards. Polar samples were spiked with 5 µl of deuterated (D4) succinic acid (1 mg/ml) and non-polar saponified extracts were spiked with 5 µl deuterated (D27) myristic acid (1 mg/ml) (Cambridge Isotope Laboratories, Tewksbury, Massachusetts, USA). The samples were dried under vacuum and derivatized with 30 µl methoxyamine hydrochloride (1 h, 40 °C) and 70 µl MSTFA (2 h, 40 °C) as previously described (Perez-Fons et al., 2014). We then injected 1 µl of the derivatized solution in splitless mode into a 7890B GC linked to a 5977A MS (Agilent Technologies, Palo Alto, California, USA). Metabolites were separated in a DB-5MS 30 m × 250 µm × 0.25 µm column (J&W Scientific, Folsom, California, USA), equipped with a 10-m guard column using a temperature gradient ranging from 70 °C to 320 °C at 10 °C/min. Helium was used as the carrier gas and the flow rate was 1 ml/min. The inlet was heated to 280 °C and the MS transfer line to 250 °C. AMDIS (v2.73) software was used for peak integration and deconvolution, and

to establish the authors' libraries for polar and non-polar metabolites as previously described (Perez-Fons et al., 2014). Metabolite quantities were normalized against the internal standards and corrected by dried weight.

Data analysis

Multivariate analysis, analysis of variance (ANOVA) and pairwise comparisons (Tukey's honest significant difference (HSD) post-hoc test and Student's *t*-test) were carried out using JMP-Pro v14.1.0. (SAS Institute, Cary, North Carolina, USA).

Results

Identification and multivariate analysis of metabolites in M37W and HC tissues under different N regimes

The combined GC-MS and UPLC-PDA analysis of leaf tissue from M37W and HC plants grown under the two different N regimes revealed the presence of 51 metabolites in the leaves of both varieties under N+ conditions but only 43 (M37W) and 48 (HC) metabolites under N- conditions. Similarly, the analysis of roots revealed the presence of 32 (M37W) and 34 (NC) metabolites under N+ conditions compared to 31 (M37W) and 33 (HC) under N- conditions (**Table S1**). These results indicate that the genotype-dependent effects of N availability on the presence or absence of particular metabolites are more prevalent in the leaves than the roots of the M37W and HC plants.

Principal component analysis (PCA) revealed that N availability had a stronger effect than genotype on the metabolic profile of the leaves (**Fig. 1**). In the PCA score plot, principal component 1 (PC1) separated the samples by N treatment regime and explained 36.7% of the variability, whereas PC2 separated the samples by genotype and explained 16.9% of the variability (**Fig. 1A**). The loading plot indicated that organic compounds lacking N (e.g., sugars and organic acids) clustered along PC2, showing a dependence on genotype, whereas N-based compounds (mostly amino acids) clustered along PC1, showing a dependence on the N treatment regime (**Fig. 1B**). The profiles of individual product classes in the four groups of plants are shown in **Fig. 1C**. In contrast, the analysis of roots revealed that genotype had a stronger effect than N availability on the metabolic profile (**Fig. 2**). The PCA score plot indicated that PC1 separated the samples by genotype and explained 43.3% of the variability, whereas PC2 separated the samples by N treatment regime and explained 20.9% of the variability (**Fig. 2A**). Sterols and sugars were associated with the clustering along PC2 (N levels), whereas sugars and phosphate pulled the separation along PC1 and were primarily responsible for the clustering of genotypes (**Fig. 2B**). The profiles of individual product classes in the four groups of plants are shown in **Fig. 2C**.

These data indicate that the enhanced production of carotenoids in the endosperm of HC maize plants has a ripple effect on the metabolic activity of leaves and roots, as previously reported for the maize embryo

(Decourcelle et al., 2015). However, the opposing PCA profiles of the leaves and roots suggest that the effect of N availability is tissue specific and should be investigated in more detail. We therefore analyzed the response of the two genotypes under optimal (N+) conditions to identify genotype-specific differences in metabolism in the absence of nutrient stress, and compared this to the response under N- conditions to determine whether the response to nutrient stress was genotype-dependent.

Genotype-dependent metabolic responses in leaves under N+ conditions

The analysis of N metabolism in the leaves revealed few differences between the genotypes under N+ conditions. Most amino acids were present in both genotypes at similar levels, although threonine was only detected in the HC leaves (**Fig. 3**). Even so there was no significant difference between the genotypes in terms of overall amino acid levels (**Fig. 1C, Table S1**). The levels of phosphate and dihydrouracil, a pyrimidine derivative, were similar in both genotypes (**Fig. 1C and 3, Table S2**).

Several organic acids were detected in the leaves of both genotypes, but the total concentration of organic acids was significantly higher in HC than M37W (**Fig. 1C**). TCA cycle intermediates (malic, aconitic and itaconic acid) were significantly more abundant in HC leaves (**Fig. 3, Table S2**) and oxalic acid, a product of ascorbate metabolism, was detected only in HC leaves (**Fig. 3, Tables S1 and S2**). We observed no significant genotype-dependent difference in total sugar levels in the leaves, although sedoheptulose was only detected in M37W and methyl-rhamnose was significantly more abundant in HC (**Fig. 1C and 3**).

We observed few genotype-dependent differences in lipid metabolism. There was a trend towards lower levels of fatty acids and higher levels of C14:0 to C18:0 monoglycerides in HC leaves, but this was not statistically significant (**Fig. 1C and 3, Table S2**).

Interestingly, total carotenoid levels were significantly lower in HC compared to M37W leaves (**Fig. 1C**). The only carotene present in both genotypes was β-carotene, and the only xanthophyll clearly detected was lutein, with significantly lower levels of both compounds in HC leaves (**Fig. 3, Tables S1 and S2**). The other xanthophylls did not separate clearly during UPLC analysis, so the concentration of 'xanthophylls' represents a mixture of all xanthophylls other than lutein (**Fig. 3, Table S2**). Several intermediates of chlorophyll metabolism were identified in both genotypes and, like the carotenoids, they were significantly less abundant in HC compared to M37W leaves both as a group and as individual products (**Fig. 3, Tables S1 and S2**).

Genotype-dependent metabolic responses in roots under N+ conditions

In contrast to the leaves, the analysis of N metabolism in the roots revealed significant differences between the genotypes under N+ conditions. The concentration of total amino acids was significantly higher in HC roots (**Fig. 2C**), particularly due to increases in the levels of serine and alanine (**Fig. 3, Table S3**).

The total concentration of organic acids was significantly lower in HC roots (**Fig. 2C**) mostly due to the depletion of the TCA cycle intermediate aconitic acid, which was 82% less abundant in HC compared to M37W roots (**Fig. 3, Table S1**). However, there was little difference in sugar metabolism between the genotypes in terms of total sugar levels (**Fig. 2C**) or the abundance of specific compounds (**Fig. 3**).

Again in contrast to leaves, we observed significant genotype-dependent differences in lipid metabolism when we compared M37W and HC roots. The total fatty acid content was higher in HC roots (**Fig. 2C, Table S3**), mainly reflecting differences in the levels of pentadecanoic and linolenic acid (**Fig. 3**), and HC roots also contained significantly greater quantities of monoglycerides (**Fig. 2C and 3, Table S3**). Although the total concentration of steroids was similar in HC and M37W roots (**Fig. 2C**), the level of β-sitosterol was significantly higher in the HC genotype (**Fig. 3, Table S3**). Carotenoids were not detected in roots of either genotype, likely due to methodological limitations to detect traces of this compounds.

Genotype-dependent metabolic responses in leaves under N- conditions

As anticipated, N- conditions had a significant effect on N metabolism in the leaves of both genotypes, but the effect was more severe in HC leaves, where the amino acid levels fell significantly lower compared to M37W (**Fig. 1C**). Alanine levels were significantly lower in HC leaves, whereas GABA, serine and threonine were not detected (**Fig. 4, Tables S1 and S2**). Phosphate levels were significantly lower in both genotypes under N- conditions (**Fig. 1C**), but again the effect was more severe in HC leaves (**Fig. 4, Table S2**). Dehydrouracil was not detected in either genotype under N- conditions (**Fig. 1C and 3, Table S1**).

In contrast to the amino acids, the total concentration of organic acids was similar under N+ and N- conditions and was therefore significantly higher in HC than M37W leaves (**Fig. 1C**). This primarily reflected the higher levels of malic, aconitic and itaconic acid, although oxalic acid was also more abundant in the HC leaves (**Fig. 4, Table S2**). In both genotypes, the total sugar content was higher under N- conditions but the difference between N+ and N- was not statistically significant (**Fig. 1C**). Sucrose and fructose were significantly less abundant in HC compared to M37W leaves (**Fig. 4, Table S2**). Glucose, which was detected in neither genotype under N+ conditions, accumulated in both under N- conditions but was more abundant in the M37W leaves (**Fig. 4, Table S2**). Sedoheptulose, which accumulated solely in HC leaves under N+ conditions, was not detected under N- conditions (**Fig. 4, Table S1**).

There was little difference in lipid metabolism between the genotypes under N- conditions, and also little difference when comparing the N+ and N- treatment groups (**Fig. 1C**). Total fatty acid levels were similar in all groups, and most individual fatty acids were present at similar levels, although arachidic and behenic acid were less abundant in HC compared to M37W leaves under N- conditions (**Fig. 4, Table S2**). The same monoglycerides detected under N+ conditions were present at similar levels in both genotypes also under

N- conditions (**Fig. 1C and 4, Table S2**). The total concentration of sterols in both genotypes was significantly lower under N- conditions but there was no significant difference between genotypes (**Fig. 1C**). However, neither campesterol nor stigmasterol was detected in HC leaves under N- conditions, so the entire sterol content was contributed by β-sitosterol (**Table S1**).

Carotenoid metabolism in the leaves under N- conditions was affected in a genotype-dependent manner. The total carotenoid content of HC leaves was similar (and low) under both conditions, whereas carotenoid levels were higher in the M37W leaves under N+ conditions but were depleted by N starvation. Hence, the total carotenoid content of leaves under N- conditions was similar in both genotypes (**Fig. 1C**). Even so, the levels of β-carotene under N- conditions were significantly lower in HC compared to M37W leaves and xanthophylls (other than lutein) were more abundant in HC leaves, although the difference was not statistically significant (**Fig. 4, Table S2**). The profile of chlorophyll and its derivatives was very similar to the total carotenoids, and accordingly there was no significant genotype-dependent difference in the total concentration of these compounds under N- (**Fig. 1C**). At the level of individual molecules, chlorophyll b was present at similar levels in both genotypes under N- conditions, chlorophyll *a* was more abundant in HC leaves (but not significantly so) and pheophytin was significantly more abundant, whereas phytol was significantly depleted (**Fig. 4, Table S2**).

Genotype-dependent metabolic responses in roots under N- conditions

The total concentration of amino acids in the HC roots was similar under N+ and N- conditions, whereas the concentration of amino acids in the M37W roots increased in response to N starvation (Fig. 2C). There were no significant differences between HC and M37W roots under N- conditions, although serine levels were significantly higher in HC roots (**Fig. 2C and 4**). Phosphate levels in the roots of both genotypes declined in response to N starvation and similar levels were detected in both genotypes under N- conditions (**Fig. 2C and 4**).

In contrast, the total concentration of organic acids in the HC roots increased under N- conditions whereas there was no significant difference between N+ and N- conditions in the M37W roots (**Fig. 2C**). The total concentration of organic acids was also higher in HC compared to M37W roots under N- conditions, reflecting significant increases in the levels of malic, aconitic and propionic acid (**Fig. 2C and 4**). Sugar metabolism was similar in both genotypes, with sugar levels in the roots increasing due to nitrogen starvation (**Fig. 2C and 4**). There were no significant genotype-dependent differences in the levels of any individual sugar.

There was no significant change in lipid metabolism in response to nitrogen starvation, and accordingly we observed a significantly higher total fatty acid content in HC roots compared to M37W roots under N-

conditions but no difference in either genotype when we compared the N+ and N- treatments (**Fig. 2C**). The difference between HC and M37W roots was primarily due to increases in the levels of pentadecanoic and linolenic acid. The total concentration of monoglycerides was also higher in HC roots compared to M37W roots under N- conditions, but this time there was also a genotype-dependent difference in the response to N starvation. In M37W roots there was no difference between the treatments, whereas the HC roots under N- conditions accumulated greater quantities of monoglycerides compared to HC roots under N+ conditions (**Fig. 2C**). This was a general response, affecting all of the individual monoglycerides we detected. Finally, the sterol content was higher in HC roots than M37W roots, again reflecting the accumulation of all the individual sterols we detected. N starvation had no significant effect on sterol levels in the HC roots, but the sterol content of the M37W roots was significantly lower under N- conditions (**Fig. 2C**).

Discussion

Genotype-dependent effects on primary metabolism of leaves and roots

Because it was previously reported that an endosperm-specific carotenoid mini-pathway engineered in HC also impacted the core metabolism of the embryo (Decourcelle et al., 2015), we were interested in determining whether similar metabolic perturbations occur in leaves and roots of HC at the vegetative stage. During vegetative growth, leaves and roots behave as sink organs utilizing the C and N assimilated for the synthesis of backbone molecules for growth and development (Hirel et al., 2001). Effects of transgene expression that affect plant phenotype and agronomic performance have been reported in rice (Ge at al., 2004), maize (Ma and Subedi, 2005; Subedi and Ma, 2007; Laserna et al., 2012; Shi et al., 2013), and soybean (Elmore et al., 2001a; 2001b; Raymer and Grey, 2003). More in-depth analyses including transcriptomics, proteomics, and metabolomics, have shown a ripple effect on core metabolism since the newly introduced pathway competes for resources with endogenous metabolic pathways (Barros et al., 2010; Decourcelle et al., 2015; Harrigan et al., 2016; Mesnage et al., 2016).

The N metabolism of HC is not markedly modified during the vegetative growth

We identified genotype-dependent differences between HC and M37W, related to N metabolism. Nitrogen assimilation requires the reduction of nitrate to ammonium, in leaves and roots (Tegeder and Masclaux-Daubresse, 2018). The reactions are catalyzed by nitrate and nitrite reductases in the cytosol and plastids, respectively (Tegeder and Masclaux-Daubresse, 2018). Ammonium is then assimilated into amino acids by glutamine synthetases (GSs), glutamine oxoglutarate aminotransferase (GOGATs) and asparagine synthetases (ASs) (Masclaux-Daubresse et al., 2010). In our study, we found very few qualitative and quantitative differences, with threonine detected only in HC leaves but not in the roots of either HC or M37W. Threonine is a product of aspartate metabolism. It utilizes oxaloacetate as precursor, additionally, serine,

glycine and threonine can be interconverted easily (Hildebrandt et al., 2015). No other amino acid derived from aspartate metabolism was detected in either HC or M37W, suggesting that threonine might be derived from the interconversion of glycine and serine and not from aspartate catabolism. Serine and glycine were detected at higher levels in HC leaves, but this difference was not statistically significant, likely due to a high interconversion rate of these amino acids to threonine. Serine was also detected in the roots of HC but not in M37W, and so was alanine. Amino acids can be translocated to roots during vegetative growth (Hirel et al., 2001). Some GS1 isoforms are found in the companion cells facilitating phloem loading for N translocation to sink organs and amino acid transporters have been characterized in Arabidopsis and *Petunia hybrida* (Tegeder and Masclaux-Daubresse, 2018). The increased levels of serine and alanine may be attributed in part to translocation of amino acids from HC leaves to roots to support root growth. Alternatively, *de novo* biosynthesis of these amino acids may also be possible, but this scenario seems less likely due to the high rate of precursors from the glycolytic pathway directed towards other metabolic pathways (please see effect on C primary metabolism below). Translocation of amino acids from leaves to roots during vegetative growth has been previously reported in Arapidopsis. Krapp et al., (2011) observed an increase in amino acids in Arapidopsis roots during N starvation. Although protein degradation might explain the response, the increased rate of root growth does not seem to support such a mechanism (Krapp et al., 2011).

Individual responses of amino acids have been reported to be genotype-specific in maize, with quantitative and qualitative differences in the amino acid profile of genetically close or distant maize lines under particular environments, such as high N availability (Cañas et al., 2017, Schlüter et al., 2012). Barros et al (2010) reported that in kernels, the amino acid response of a Bt maize and an herbicide-resistant variety differed considerably when compared to the same isogenic counterpart. L-glutamine and valine were differentially regulated in Bt maize in contrast to tyrosine and L-tryptophan in the herbicide-resistant variety. However, the profile of differentially regulated amino acids turned out to be a one-year effect, and the authors concluded that the environment was the most important factor which overrode any possible effects of genotype. Manneti et al., (2006), also reported altered levels of amino acids in Bt maize kernels relative to the non Bt. Mesnage et al. (2016), reported that at least 20 amino acids were differentially regulated in the kernels of a glyphosate-tolerant maize compered to its parental line. Here, we observed few differences between the amino acid profiles of HC and M37W on N metabolism in leaves and roots.

The endosperm-specific metabolic intervention caused divergent patterns of sugar and lipid accumulation in vegetative tissues

Strong metabolic readjustments occurred in the primary metabolism of HC, with characteristic patterns of accumulation for C-containing metabolites. Both genotypes, employed glucose as the main source of energy

and C skeletons for intermediate synthesis since glucose was not detected in the leaves, likely due to its depletion. Upstream the glycolytic pathway, a high flux of intermediates was diverted towards the synthesis of methyl-rhamnose and not sedoheptulose as in M37W. Free sedoheptulose functions as C storage in plants and can be later incorporated into the pentose phosphate pathway (Cowan, 2017), whereas rhamnose, which is an important cell wall component, is predominantly stored as a glycoside (Wang et al., 2008). The increased precursor demand for the synthesis of these metabolites reduced flux towards the end of the glycolytic pathway, impacting carotenoid synthesis. No previous quantification of these compounds had been done in the leaves of HC, because the carotenogenic mini-pathway introduced is endosperm specific, and thus, no significant changes were expected to occur in the leaves, (Zhu et al., 2008; Decourcelle et al., 2015; Zanga et al., 2016). Carotenoids in HC leaves might be downregulated as part of a trade off with the synthesis of sterols and lipids, which were not differentially regulated in HC, likely because sterols and fatty acids are incorporated into membranes as structural components during vegetative growth (Schaller, 2004; Ferrer et al., 2017). Similarly, the strong upregulation of fatty acids and monoglycerides in roots might be due to the high translocation rate of sucrose from leaves. Carbohydrates are synthetized in the leaves and sucrose is translocated to roots to support root growth during the vegetative stage (Zakhartsev et al., 2016). Although the levels of sucrose in HC leaves were not statistically different from those in M37W, HC showed a trend towards lower levels of sucrose (20% less).

Strong upregulation of energy metabolism is tissue specific

The most interesting metabolic adjustments in leaves were marked by a strong upregulation of the organic acids aconitate, itaconate and malate, which may reflect an imbalance in energy metabolism. Organic acids play multiple roles in plants, such as in the maintenance of the redox balance, production and consumption of ATP, and as pH regulators (Igamberdiev and Eprintsev, 2016; Ludwig, 2016; Igamberdiev and Bykova, 2018). In contrast, only aconitic acid appears to be differentially regulated in HC roots (82 % less). The reduced levels of this TCA-cycle intermediate might be related to an increase in propionic acid, which required high flux of succinyl-CoA derived from the TCA cycle. Aconitate, is an intermediate in the formation of isocitrate from citrate in the TCA cycle. We were not able to distinguish between *cis* and *trans* isomers, but it is likely that we identified *trans*-aconitate. The c*is* isomer appears as a low concentration intermediate, whereas *trans*-aconitate is a more stable isomer and can be stored in plant tissues (Igamberdiev and Eprintsev, 2016). Therefore, propionic acid might be produced at the expense of aconitic acid in roots. Similar alterations in TCA cycle intermediates have been reported in Bt-maize kernels, in which citric acid levels increased whereas levels of fumaric acid decreased (Piccioni et al., 2009). In contrast, a metabolic analysis in kernels of an herbicide-resistant maize showed that levels of organic acids from the early TCA cycle were reduced, but organic acids from the later part of the cycle increased (Mesnage et al., 2016). In

HC kernels, however, no changes in organic acids were observed relative to M37W (Decourcelle et al., 2015).

The accumulation of aconitate in HC leaves suggests a reduction in the flow of intermediates downstream the TCA cycle. In C_4 plants, malate is produced during photosynthesis and functions as shuttle for C and reducing equivalents between the mesophyll and the bundle sheath cells (Stitt and Zhu, 2014; Ludwig, 2016). The increased pool of malate in HC leaves (123 % more than M37W), was likely derived from photosynthesis. This scenario, however, might involve a low decarboxylation rate occurring in the bundle sheath cells, where malate is decarboxylated to pyruvate to provide CO_2 for the synthesis of carbohydrates (Ludwig, 2016). Reduced decarboxylation of malate might partially explain the reduced pool of sucrose and fructose in HC, since less CO_2 would be available for sugar synthesis in HC compared to M37W. It is unclear, however, what drives reduced malate decarboxylation rate. A possible explanation would involve an impairment in the 2-oxoglutarate\malate transporter localized in the chloroplast membrane of the bundle sheath cells (Taniguchi and Miyake, 2012). Alternatively, decreased activity (or levels) of the NADP-malic enzyme could be involved. In this scenario, the chloroplast would need to balance the production and consumption of energy (ATP) and reducing equivalents (NADPH) by dissipating the intermediate in excess (Kramer & Evans, 2011). The excess of malate would then be exported to the cytosol (Scheibe, 2019). Malate in the cytosol can be reconverted into oxaloacetate to generate NADH for nitrate reduction, it can be stored in the vacuole, it can be metabolized in the mitochondria to synthesize ATP (Scheibe, 2004) or it may be translocated through the phloem to the roots (Touraine et al., 1992).

Chlorophyll content is markedly reduced in HC leaves

Upregulation of aconitate came also at the expense of decreasing the availability of precursors for chlorophyll synthesis. The TCA-cycle intermediate 2-oxoglutarate is a precursor of glutamate, an amino acid required for chlorophyll synthesis through the intermediate 5-aminolevulinate (Gough et al., 2003). Interestingly, an earlier evaluation carried out with HC plants showed no differences between HC and M37W in terms of chlorophyll content (Zanga et al., 2016). In the earlier study, however, chlorophyll content was only estimated *in situ* using a SPAD-520 portable chlorophyll meter and no quantitative analysis was performed (Zanga et al., 2016). Although an impairment of the photochemical reactions that produces ATP and NADPH during photosynthesis would be expected, as chlorophyll is an important component of the reaction center of the photosystem II (PSII), no significant effects on the effective PSII quantum yield were reported for a maize line with reduced chlorophyll content (Schlüter et al., 2012). Similarly, Zanga et al. (2016) reported no significant differences in the photosynthetic rate and photosynthesis duration between HC and M37W. This suggest that the photosynthetic capacity of HC was not impaired by the decrease in chlorophyll.

Nitrogen-dependent effects on C and N primary metabolism

Effect of limited N availability on N and P metabolism

Limited N availability impairs plant growth and metabolism (Amiour et al., 2012, Schlüter et al., 2012; 2013; Baek et al., 2019). Although HC and M37W shared common metabolic responses to low N availability, the two genotypes can be distinguished due to changes in specific metabolites. The synthesis of amino acids is strongly affected by reduced N availability, because in plants, nitrate and ammonium are reduced to amino acids (Tegeder and Masclaux-Daubresse, 2018). During vegetative growth, the amino acid profile of HC leaves was strongly affected by limited N supply, with significant qualitative and quantitative changes in all amino acids detected, but remarkably, no differential changes were observed in roots. In contrast, only two amino acids were differentially regulated in M37W leaves, but the effect might be related with an increase of these amino acids in roots. These results suggest that during low N availability, the translocation of amino acids from leaves to roots in HC is reduced. Similarly to M37W in Arabidopsis, N starvation reduced the amino acid pool in the shoots, whereas these increased in the roots (Krapp et al., 2011). The authors suggested that the translocation of amino acids from shoots to roots under limited N supply, allowed root growth to continue under these circumstances (Krapp et al., 2011). In maize, Schlüter et al. (2012), reported amino acid decrease in maize leaves during vegetative growth under short and long N starvation, with characteristic individual responses of amino acids shown by different genotypes. Alanine, serine, glycine, aspartate, asparagine, threonine, and glutamate were significantly downregulated in the A188 genotype, whereas the same amino acids were either upregulated or remained unaltered in the B73 genotype (Schlüter et al., 2012). Similarly, Amiour et al., (2012) observed that 18 amino acids (out of 22) were decreased from 4- to 37-fold during the vegetative growth of maize plants under low N. Similar results have been observed also in tobacco (Fritz et al., 2006), and tomato (Gil et al., 2020). These studies, however, did not investigate changes in roots.

Krapp et al. (2011) reported up to 80 % decrease in phosphate in response to limited N supply in Arabidopsis shoots, but interestingly, in roots, phosphate increased up to 300%. In maize, it has been reported that phosphate is the metabolite with the strongest increase in leaves under low-N (Schlüter et al. 2012, 2013) and similar results were reported for radish shoots after 7 days of N starvation (Baek et al., 2019). However, in the study of Schlüter et al. (2012), the authors suggest that it cannot be ruled out that the high phosphate concentration in leaves was caused by comparably high inorganic phosphate supply in the nutrient solution and the length of the experiment.

Effect of limited N availability on sugar metabolism

Carbon and N metabolism are highly interconnected (Fait et al., 2018, Stitt and Krapp, 1999) with C molecules functioning as skeletons for the formation of N-containing molecules (Hirel and Lea, 2001). A strong correlation between reduced N supply and increased availability of C-containing primary metabolites such as sugars, sugar alcohols, lipids, and organic acids has been reported in Arabidopsis, maize and radish sprouts (Krapp et al., 2011; Schlüter et al., 2012; 2013; Baek et al., 2019). M37W and HC leaves had divergent patterns of accumulation for these groups of metabolites. Glucose was the only soluble sugar increased in both genotypes, whereas as for other glycosides, sugars, and sugar alcohols were either increased or decreased. In roots, however, the sugar profiles were more similar. In Arabidopsis and radish, an increase in the content of soluble sugars was measured in the shoots and roots after 2 days of N starvation. Glucose and fructose increased in both shoots and roots (Krapp et al., 2011), and in radish sprouts similar results were found for shoots (Baek et al., 2019). For these species, the response of these sugars has been correlated with a decrease of amino acids, which would decrease the demand for C skeletons for amino acid biosynthesis (Obata and Fernie, 2012, Baek et al., 2019). Following this line, the increased levels of glucose and fructose we measured in HC and M37W in response to low N availability might be related to the decrease of several amino acids, which reduced the demand for C skeletons provided by glycolysis. However, our results, are inconsistent with the response observed in leaves of maize plants grown under limited N supply. Schlüter et al. (2012; 2013) reported decreased levels of glucose and fructose, and similarly Amiour et al. (2012). In these studies, however, other sugars and sugar alcohols either increase or decrease, for instance, sugars from the raffinose group were increased (Schlüter et al., 2012; 2013), and lactose, mannose, pentitol and sorbitol were decreased (Amiour et al., 2012).

Effect of limited N availability on lipid metabolism

Decrease in the content of fatty acids was observed in the leaves of both genotypes, although M37W had far more fatty acids decreased. Since fatty acid synthesis requires successive incorporations of acetyl-CoA in the plastid (Ohlrogge and Browse, 1995), downregulation of these metabolites would decrease the demand of assimilates. The decrease in medium- to long chain fatty acids (up to C26) in M37W partly coincides with the findings of Schlüter et al. (2012; 2013), in which fatty acids of up to C18 were downregulated under low N, whereas no changes were observed for longer fatty acids. In contrast, the effect of limited N on the fatty acid composition of HC was minimum, with changes only in pentadecanoic acid (increase) and palmitic acid (decrease), which indicates a low demand for acetyl-CoA, which in turn might have contributed to the increase in fructose and glucose availability. In roots, the response of HC, but not M37W, coincides with the findings of Krapp et al. (2011) in Arabidopsis, in which linolenic acid was upregulated in the roots under N starvation.

The increased availability of soluble sugars under N limitation influenced other metabolic pathways that also use glycolytic intermediates as precursors. Free sterols, which are synthesized through the mevalonate pathway from isopentenyl diphosphate using pyruvate as a precursor (Ferrer et al., 2017), decreased in both genotypes, although the sterol profile under low N differed qualitatively in the two genotypes. Stigmasterol and β-sitosterol were detected in M37W, whereas in HC, stigmasterol was not detected. In radish sprouts growing under contrasting N levels, however, no distinguishable difference was found in the levels of sterols (Baek et al., 2019). Other studies in maize at the vegetative stage, however, did not report changes in sterol content under low N (Schülter 2012; 2013; Amiour et al., 2012).

Effect of low N supply on organic acids

Surprisingly, the metabolic signature of organic acids in HC leaves was not altered under low N supply. In M37W the impact was minimal with only a significant decrease in maleic acid, although a trend towards decrease was also observed for itaconic and malic acids. We measured a decrease of organic acids of the TCA cycle in M37W roots, but an increase in HC. This response in HC is different from the general trend reported for maize grown under low N, in which a decrease in the amount of several TCA cycle organic acids occurs (Amiour et al., 2012; Schlüter et al., 2012). Malate concentration in leaves is severely reduced under low N (Amiour et al., 2012; Schlüter et al., 2012), as well as aconitate, citrate and fumarate (Schlüter et al., 2012). Schlüter et al. (2012) correlated the strong response of malate with the decrease in C and N assimilation, which would reduce the demand for malate as the main C_4-specific shuttle in maize and as a pH regulator during nitrate reduction (Tschoep et al., 2009; Schlüter et al., 2012). Similarly, Baek et al. (2019) reported a significant decrease in TCA cycle organic acids in radish sprouts under N starvation. In contrast, in Arabidpsis leaves and roots, organic acids show an increasing trend in response to N starvation, although amino acids decreased (Krapp et al., 2011). The increased pool of malate was assumed to contribute to the synthesis of amino acids through the TCA cycle, which would provide the 2-oxoglutarate moiety. However, in our study amino acids decrease in both genotypes, but far more in HC. Therefore, it is unlikely that organic acids were contributing to amino acid synthesis.

Effect of low N supply on chlorophyll and carotenoid content

Chlorophyll content in leaves is an indicator of N content in plants (Zhao et al., 2005). Photosynthesis is one of the first process to be impacted by low N supply since the later results in a decline in the content chlorophyll (Zhao et al., 2005). Our results are consistent with the finding of Zanga et al, (2016), in which the levels of chlorophyll in HC and M37W were lower than in non-fertilized plants. Similar results have been reported in other studies with maize (Amiour, et al., 2012; Schlüter et al., 2012), wheat (Heyneke et al., 2017) and radish sprouts (Baek et al., 2019). Our findings suggest that HC might have lower amount of photosynthetic

apparatus of which chlorophyll is part. Zanga et al., (2016) reported that the duration of HC photosynthesis was similar for fertilized and non-fertilized plants. Schlüter et al. (2012) reported that the effective PSII quantum yield of maize leaves was only slightly affected under low N, despite the reduced levels of chlorophyll. The authors indicated that the remaining PSII reactions centers were intact suggesting that the remaining PSII in HC might be fully functional.

In addition to reduced chlorophyll levels, reduced carotenoid content as a response to N limitation has been reported in rice, sorghum. sweet potato leaves, and radish sprouts (Huang et al., 2004; Zhao et al., 2005; Wei et al., 2015; Baek et al, 2019). In our study, the carotenoid content in leaves of M37W was also found to be severely affected by low N availability. The reduction of carotenoids might be related to the decrease in chlorophyll content, which might also reduce the demand for accessory pigments (Baek et al, 2019). The decrease in sterols and carotenoids in M37W might be interconnected, since there is a simultaneous translocation of prenyl diphosphates between the mevalonate pathway in the cytosol and DXS pathway in the plastids (Bick and Lange, 2003). Remarkably, carotenoids were not altered by low N availability in HC leaves. However, even under high N the chlorophyll and carotenoid levels in HC were lower than those in M37W, and therefore the demand for accessory pigments was lower and could be satisfied even at low N.

Interactive effects of genotype and N supply on primary metabolism of leaves and roots

An earlier evaluation between HC and M37W found them indistinguishable in terms of agronomic performance under high as well as low N (Zanga et al., 2016). Our study, however, represents a more in-depth analysis of the metabolic readjustments experienced by the vegetative tissues of HC because of the introduced endosperm-specific carotenogenic mini-pathway. Despite their common response under limited N supply, there were specific patterns of metabolite accumulation in HC vegetative tissues that can be attributed to an interactive effect of genotype and N supply.

Interactive effects of genotype and environmental stress on the amino acid content were reported in Bt rice. In response to drought amino acid content in Bt rice increased relative to the isogenic counterpart (Jiang et al., 2018). In our study, a shift in the allocation pattern of N into amino acids was observed in leaves of HC and M37W. However, the fact that amino acids from two different metabolic pathways, namely alanine and GABA, were more reduced in HC (45% less than in M37W), suggests strong control mechanisms operating at different points of HC metabolism. Multiple studies reported high stability in the content of glutamate even under N starvation since it has an important role as the amino group donor for the synthesis of GABA and other amino acids (Fritz et al., 2006, Krapp et al., 2011). Glutamate was below the limit of detection in HC leaves and roots, but the high levels of aconitate and itaconate, suggest a strong regulation in the TCA cycle that keeps levels of these organic acids high even at the expense of precursors, such as 2-oxoglutarate,

utilized in the synthesis of essential amino acids (Hildebrandt et al., 2015). In M37W, however, all TCA cycle intermediates detected under high N were depleted, likely to support amino acid synthesis (Krapp et al., 2011; Hildebrandt et al., 2015). Downregulation of amino acids in HC roots was minimal even under low N. with serine levels remaining unaltered. Serine, glycine, and threonine can be easily interconverted (Hildebrandt et al., 2015), and threonine can enter into the TCA cycle after its conversion to oxaloacetate (Kirma et al., 2012). The use of amino acids as a source of C skeletons and energy occurs especially under conditions of low carbohydrate availability (Araújo et al., 2011, Kirma et al., 2012)

The high accumulation of malate, aconitate, and itaconate also suggests a strong control mechanism to deliver precursors derived from glycolysis towards the TCA cycle, since downregulation of these acids is a common response to N deficiency observed in maize (Amiour et al., 2012; Schlüter et al., 2012, 2013), and it was also observed in M37W in our experiments. This particular response observed in HC was accompanied with a strong decrease of campesterol, β-carotene and a number of long-chain fatty acids, all of them requiring precursors from the last steps of the glycolytic pathway, although their synthesis is compartmentalized in different structures. Levels of organic acids were high also in HC roots. Krapp et al., (2011) also observed high accumulation of organic acids in both shoots and roots of Arabidopsis after 10 days of N starvation. Malate was considered to contribute to the elevated levels of GABA found in roots, but contribution of malate in amino acid synthesis seems unlikely to be the reason in our experiments, since GABA was not detected in the roots of either HC or M37W.

The flow of precursors was also regulated in the early reactions of glycolysis, since methyl-rhamnose levels decreased, but interestingly, in addition to TCA acid cycle, oxalic acid levels were not reduced under low N. Zhang et al. (2005) reported an increase in oxalic acid content with increasing nitrate levels in spinach, thus, oxalic acid content might have been expected to decrease in HC under low N but instead it remained unaltered. The strong regulation of oxalic acid might be linked to ion balances or calcium regulation (Nakata, 2003; Franceschi and Nakata, 2005; Igamberdiev and Eprintsev, 2016). Oxalate can be present in soluble form or as calcium oxalate crystals. The latter enables the plant to balance calcium levels in the cells by its storage in vacuoles, thus preventing the increase of calcium levels in other compartments (Franceschi and Nakata, 2005). Alternatively, as shown in Arabidopsis, oxalate can be reincorporated in metabolism through its transformation to oxalyl-CoA by an oxalyl-CoA synthetase (Foster et al. 2012, Igamberdiev and Eprintsev. 2016).

Soluble sugars are key metabolites in the provision of C skeletons and energy production required in the synthesis of different metabolites. Although sugars decreased in both genotypes, in HC the levels of sucrose, fructose and glucose decreased even further, up to 70% less compared to M37W. Under high N supply, differences in sugar levels between in HC and M37W were not statistically significant, but under low N

availability differences became statistically significant. Differences in soluble sugars were consistently observed in the leaves between Bt maize and its near isogenic line grown under high N, although the trend was towards increase (Barros et al., 2010). In HC roots, however, sucrose levels were similar to M37W. Likely there was high translocation of sucrose from leaves to roots, and it might have contributed to the high levels of medium- and long-chain fatty acids, as well as monoglycerides found in HC roots.

It is difficult to understand the metabolic readjustments in HC leaves and roots in connection with the engineered carotenoid biosynthesis in the endosperm as we had earlier confirmed absence of transgenic mRNA in leaves and roots. Most studies on metabolic disturbances caused by the direct insertion of exogenous genes focus on Bt or herbicide-resistant maize, in which the engineered trait is expressed constitutively during the life cycle of the plant and can be attributed to the competition for limiting resources between endogenous and heterologous pathways. In kernels of Bt maize, levels of sugars and sugar alcohols were higher compared to its isogenic counterpart, and no differences were found in organic acids (Barros et al., 2010). Similarly, Manneti et al. (2006) reported altered levels of amino acids in kernels of Bt maize relative to the non-Bt maize. In contrast, in kernels of an herbicide-resistant maize, organic acids from the early TCA cycle (citrate) increased, but organic acids from the later part of the cycle (malate) decreased (Mesnage et al., 2016). Similarly, sugars were lowered in Bt maize kernels compared to the non Bt, citric acid increased but no other organic acid decreased (Piccioni et al., 2009). These studies suggest that changes in the metabolic flow of TCA cycle and carbohydrate metabolism are consistently observed in these plants, but the origin of these alterations was not elucidated. Additionally, these studies were conducted with plants grown under an optimal N supply, and therefore, the extent to which their metabolism could be readjusted when N is limited remains unknown. In HC, the engineered carotenogenic pathway is not expressed constitutively, and therefore it is unlikely that the metabolic adjustments observed in HC at the vegetative stage arise due to resource competition between the endogenous and introduced pathways.

Reprogramming of the primary C metabolism in HC during vegetative growth is consistent with the increased precursor demand during grain filling

Our results strongly suggest that a trade-off occurred between the synthesis of organic acid intermediates of the TCA cycle and the synthesis of carotenoids and chlorophylls in HC. Although compartmentalized in different cellular structures, the biosynthetic pathways leading to these metabolites compete for common precursors derived from glycolysis in the cytosol. Our results are, however, a snapshot of the metabolic status of HC plants at a specific developmental stage. The metabolic profile of plants is known to differ considerable at different developmental stages, due to the shifting of leaves from sink to source organs during the kernel-filling period (Hirel et al., 2005a; 2005b, Krapp et al., 2011; Amiour et al., 2012; Heyneke et al., 2017). Amiour et al. (2012) observed divergent patterns of metabolite accumulation between maize

leaves during vegetative growth and kernel filling, which were characterized by a strong depletion of soluble carbohydrates and TCA cycle intermediates such as sucrose and aconitate, respectively. Similarly, in wheat leaves, glucose, fructose, and amino acid levels decreased during grain filling (Heyneke et al., 2017). It could be expected then that the metabolic profile of HC changes at later developmental stages, mainly due to a reallocation of the metabolites accumulated during vegetative growth that will support grain synthesis. Thus, at grain filling, the expression of the endosperm-specific carotenoid pathway in HC is expected to impact primary metabolism, since precursors for the introduced pathway need to compete with native pathways (Sandman, 2001).

Since the extended carotenoid production in the endosperm is supported by an increased precursor supply, our results allow us to propose candidates for future remobilization during grain filling from source leaves to sink organs. Although sucrose is the main exported sugar (Zakhartsev et al., 2016), the low content of this metabolite in HC leaves would not support the increased demand of precursors needed in the kernel. This is in line with the findings of Decourcelle et al, (2015), in which the sucrose content in kernels was high but the activities of sucrose synthase (EC 2.4.1.3) and UDP-glucosyl transferase (EC 4.1.2.13) were low, suggesting a minor contribution of sucrose to glycolysis in the kernel. The same authors suggested that the C supply in HC kernels could then be provided by the import of glucose and fructose. In our study, glucose was found to be depleted at high N and fructose was decreased in HC compared to M37W, thus, their import to kernels might be limited, although it cannot be excluded. Alternatively, based on our results, we suggest that the excess of malate, aconitate and itaconate accumulated in leaves during the vegetative growth of HC could be reallocated to kernels during grain filling and used as an alternative source of C and energy. High content of malate and aconitate have been reported in the phloem exudates of wheat and maize (Palmer et al., 2014; Yesbergenova-Cuny et al., 2016; Heyneke et al., 2017) indicating that TCA-cycle intermediates can be transported to developing seeds and used to provide C skeletons and energy (ATP and NADPH) for the synthesis of other metabolites (Araujo et al., 2011; Heyneke et al., 2017). Indeed, malate is known to be a good source of C to support the synthesis of fatty acids in castor endosperm, which is known to accumulate high levels of fatty acids (Smith et al., 1992). More recently, a metabolomic and fluxomic study in maize showed that malate can be actively use as a source of carbon, energy and reducing power in fatty acid synthesis in the maize embryo (Cocuron et al., 2019). Thus, in our model malate is decarboxylated in the cytosol and provides the extra pyruvate needed to support the increased demand of precursors for carotenoid, sterol, and fatty acid synthesis. Aconitate and itaconate enter the TCA cycle and provide the additional ATP and NADPH (Araujo et al., 2011; Heyneke et al., 2017). Indeed, Decourcelle et al., (2015) suggested than an alternative route for pyruvate formation that by-passes pyruvate kinase catalysis could be taking place in HC kernels. Although their model was based on the use of imported

glucose and fructose, they further suggested than a cytosolic NADP-malic enzyme might be involved in the alternative route, since a NADP-malic enzyme has been reported to be exclusively expressed in maize embryos and roots (ZmCytNADP-ME) (Detarsio et al., 2008). Thus, the two models are not mutually exclusive.

Transgenerational priming induced by metabolic reprogramming

Decourcelle et al. (2015) demonstrated that the expression of carotenogenic genes in the endosperm of HC led to metabolic changes also in the embryo. The authors suggested that the increased demand of metabolite precursors in the kernels due to the extended biosynthetic capacities was provided by the import of fructose and glucose from source leaves Our results show that the expression of carotenogenic genes in the endosperm elicited metabolic readjustments in vegetative tissues well in advance of flowering, pollination, and seed formation. The differentially accumulated molecules are primary metabolites that can be channeled to any direction to supply C skeletons and ATP, and therefore can play a pivotal role in the C supply during seed development. Vegetative tissues of HC plants do not express the engineered carotenogenic mini-pathway and expression of transgenes at the mRNA level was neither observed in leaves nor roots, which suggests that the differentially expressed metabolic profile could not have arisen from metabolite competition between endogenous and the engineered pathways. Alternatively, the metabolic response in HC was initiated well in advance of seed formation. This suggests that vegetative tissues were primed for the upcoming demand of metabolite precursors imposed by seed development. The experience of a stimulus that indicates future stress can prime or prepare plants for an improved response when the new challenge is encountered (Bruce et al., 2007; Hilker et al., 2015). Several biotic and abiotic factors function as elicitors of primed responses in plants, such as herbivore or pathogen attack, and environmental factors such as drought, high temperature and cold (Bruce et al., 2007; Kinoshita and Seki, 2014; Pastor et al., 2014). Schwachtje et al., (2019) postulated that metabolic disturbances in themselves can function as priming elicitors. Changes in metabolite concentrations, metabolic fluxes or metabolite ratios that become apparent in the sizes of the metabolic pools, implies the transition of an initial metabolic state into a stress-induced state (Schwachtje et al., 2019). Induced changes in the metabolic profile are believed to represent a metabolic imprint or stress memory of prior disturbances and these can prime plant responses to subsequent stresses (Gamir et al., 2014; Hilker et al., 2015; Schwachtje et al., 2019). The underpinning mechanisms would include epigenetic modifications, changes in gene expression, changes at the physiological and metabolic levels, (Bruce et al., 2007; Sahu et al., 2013; Hilker et al., 2015; Turhut-Kara et al., 2020). These modifications could be transmitted to the next generations that are likely to experience the same conditions through the germ cells (Galloway and Etterson, 2007; Paszkowski and Grossniklaus, 2011; Holeski et al., 2012; Jablonka 2012; Chen and Arora, 2013; Saavides et al., 2016; Zheng et al., 2017;

Weinhold 2018). Under this scenario, it is likely that the stress-induced changes in the metabolic profile imposed by the increased demand of metabolic precursors during seed formation in the original generation could function as a priming elicitor and was inherited to the next generations, that were likely to experience the same metabolic disturbances. Slaughter et al., (2012) showed that the primed state of Arapidopsis plants induced by β-amino-butyric acid (BABA) against the bacteria *Pseudomonas syringae* pv *tomato* was still functional in the next generation without additional treatment. Zhang et al. (2017) reported that multi-generational drought imposition to rice across 11 generations, led to the plant's adaptation to drought stress. It was confirmed that DNA methylation patterns were affected by multi-generational drought stress. The authors suggested that the epigenetic variations observed played an important role in the long-term adaptation of the plants by direct participating in stress-responsive patterns (Zhang et al., 2017). Priming of plant responses, however, requires a temporal gap between the perception of the priming elicitor and the triggering stress, known as the recovery or priming phase (Hilker et al., 2015). Pastor et al., (2014), showed that during the recovery phase, primed plants generate and accumulate precursor molecules that can be released during the triggering event. A metabolomic analysis of Arabidopsis primed with BABA, showed a boost of primary metabolism through the increased accumulation of intermediates of the TCA cycle during the priming phase (Pastor et al., 2014). It is likely then that the metabolic readjustments observed in leaves and roots of HC, which included an increased pool of intermediates of the TCA cycle, were part of a transgenerational priming induced during the increased demand of precursors during seed formation and inherited through the germ cells. Bruce et al. (2007) pointed out that although priming increases overall plant performance, trade-offs are likely to occur and could compromise vital physiological processes such as photosynthesis. In our case, the metabolic priming observed in HC came at the cost of a trade-off between chlorophyll and carotenoid synthesis and the accumulation of organic acids, which also impacted sugar content in leaves and roots.

Conclusions

We conducted an in-depth analysis of the metabolic status of HC vegetative tissues to investigate the metabolic readjustments cause by the engineering of an endosperm-specific carotenogenic mini-pathway. Differences were observed between HC and M37W mainly in the pattern of accumulation of organic acids, chlorophyll, and carotenoids. HC accumulated significantly higher levels of malate, aconitate and itaconate in leaves but reduced levels of chlorophylls and carotenoids. There was also an increase of aconitate in roots. Further readjustments in the primary C and N metabolism occurred when N availability was limited. But again, organic acids were strongly increased in HC leaves compared to M37W. Interestingly a strong decline in soluble sugars in HC was seen under low N. We, therefore, propose a model in which malate, aconitate, and itaconate accumulated during vegetative growth of HC, is further translocated to seeds when

699 leaf and root tissue shift from sink to source organs at grain filing (Amiour et al., 2012). The reprogramming
700 of the C primary metabolism in leaves and roots of HC could have arisen as part of a transgenerational
701 priming induced by the stress imposed by the increased demand of metabolic precursors during seed
702 formation in the original generation (Schwachtje et al., 2019). The accumulation of organic acids during the
703 priming phase (Pastor et al., 2014) can therefore contribute to the increased demand of pyruvate for the
704 extended synthesis of carotenoids in the endosperm and fatty acids and sterols in the embryo (Araújo et al.,
705 2011, Krapp et al., 2011).

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