Research review

Why do plants blush when they are hungry?

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Summary

Foliar anthocyanins, as well as other secondary metabolites, accumulate transiently under nutritional stress. A misconception that only nitrogen or phosphorus deficiency induces leaf purpling/reddening has led to overuse of fertilizers that burden the environment. Here, we emphasize that several other nutritional imbalances induce anthocyanin accumulation, and nutrient-specific differences in this response have been reported for some deficiencies. A range of ecophysiological functions have been attributed to anthocyanins. We discuss the proposed functions and signalling pathways that elicit anthocyanin synthesis in nutrient-stressed leaves. Knowledge from the fields of genetics, molecular biology, ecophysiology and plant nutrition is combined to deduce how and why anthocyanins accumulate under nutritional stress. Future research to fully understand the mechanisms and nuances of foliar anthocyanin accumulation in nutrient-stressed crops could be utilized to allow these leaf pigments to act as bioindicators for demand-oriented application of fertilizers. This would benefit the environment, being timely due to the increasing impact of the climate crisis on crop performance.

Anthocyanins as indicators of nutrient stress

Most people have experienced it themselves – blushing in stressful situations, out of embarrassment, shyness or shame. This involuntary reddening of the face is activated by the sympathetic nervous system as part of the 'fight or flight' response (Hofmann *et al.*, 2006). Analogically, plant leaves turn red or purple under stressful conditions such as cold temperatures, high light or nutritional imbalances. Being unable to flee, terrestrial plants resist stress through metabolic changes. A change in leaf colour is often caused by the accumulation of anthocyanins. Anthocyanins, water-soluble vacuolar pigments, are a class of flavonoids with large chemical diversity and are synthesized in the cytosol via the phenylpropanoid pathway (Fig. 1a,b; Koes *et al.*, 1994; Vogt, 2010; Li *et al.*, 2019). Their general structure consists of a flavylium ion backbone with variable numbers of hydroxyl and methoxy groups. Their synthesis from the precursor phenylalanine involves several intermediary enzymatic steps (Fig. 1b; Given *et al.*, 1988; Zhang

et al., 2014). Conjugation with sugars and acylation with organic acids increase the complexity of these secondary metabolites, conferring different properties such as colour, stability and bioactivity (de Pascual-Teresa & Sanchez-Ballesta, 2008).

Leaf reddening caused by nutrient deficiency occurs in many diverse species (Fig. 1c–i) and has been described extensively (Table 1; Bergmann, 1993). In agricultural practice, this is usually, but in some cases erroneously, ascribed to a lack of nitrogen (N) or phosphorus (P). This can lead to and overuse of fertilizers with negative impact on the soil and groundwater, our major drinking water source, as well as being a waste of resources and energy. Here, we point out that anthocyanin synthesis is a more common symptom also occurring in response to several other nutrient deficiencies (Table 1).

An overview of the effects of N and P deficiency on the expression of all major enzymes of the anthocyanin pathway was presented by Lillo *et al.* (2008) for leaves of Arabidopsis. Both deficiencies upregulate gene expression of enzymes required during early (e.g.
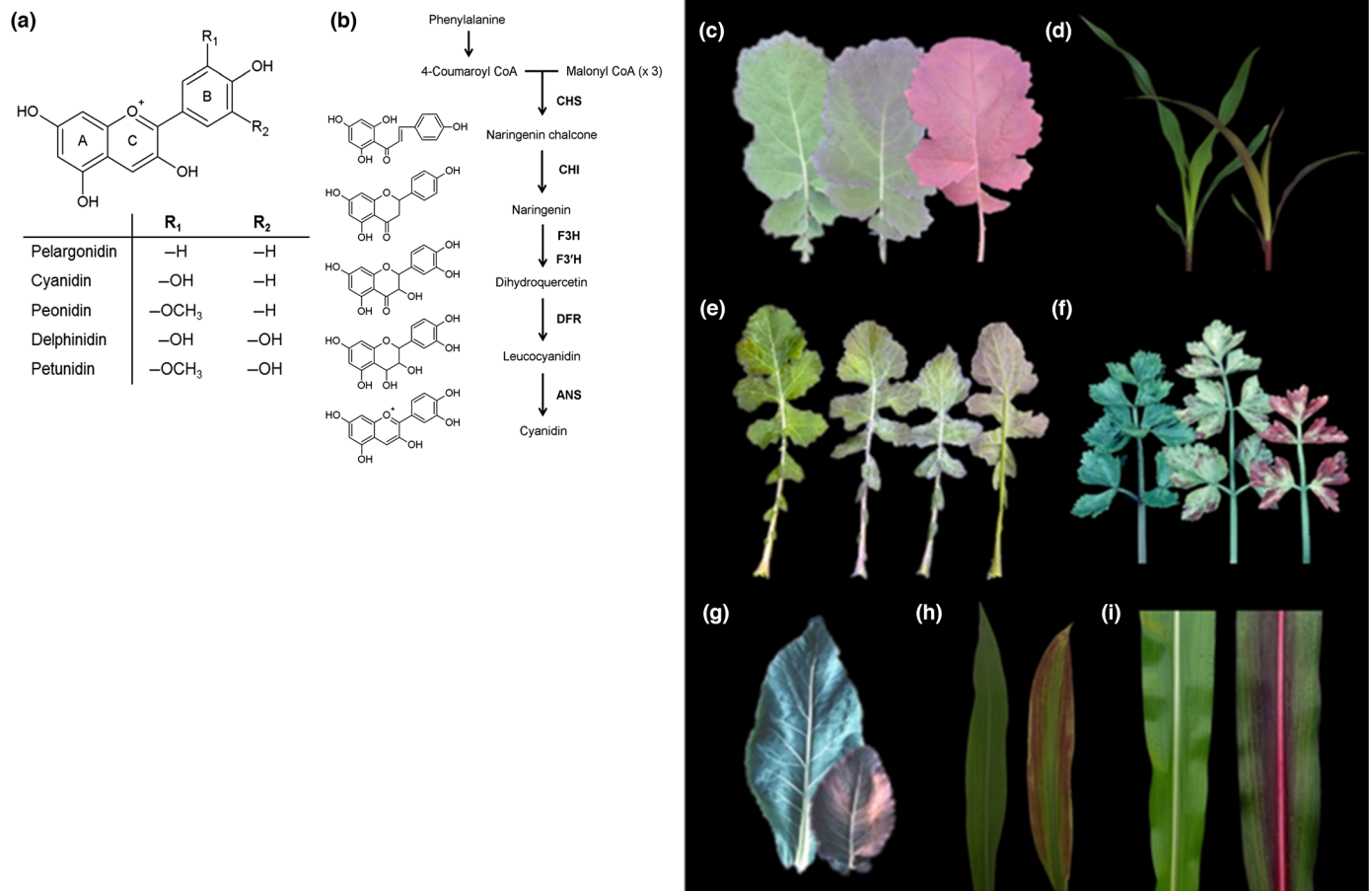
Fig. 1 Nutrient deficiency-induced anthocyanin accumulation in leaves. Anthocyanin accumulates from the activity of enzymes in the flavonoid pathway (a, b) and is elevated in leaves in response to nutrient deficiency. (c) Rapeseed (*Brassica napus*) grown with low nitrogen (N) levels in the soil. Left to right: young to old leaves of the same plant. (d) Maize (*Zea mays*) seedlings grown with optimal (left) or low (right) N levels in the nutrient solution. (e) Rapeseed showing signs of sulphur (S) deficiency. Left to right: control with optimal S supply (adaxial face), adaxial or abaxial face of leaves with limited S in the nutrient solution (adapted from D'Hooghe *et al*., 2013 and modified (background removed, elements rearranged)). (f) Celery (*Apium graveolens*) leaves with increasing symptoms of magnesium (Mg) deficiency. Left to right: decreasing levels of Mg in the nutrient solution. (g) Leaves of cauliflower (*Brassica oleracea* var. *botrytis*) grown with optimal (left) or low (right) phosphorus (P) in the nutrient solution. (h) Maize with optimal (left) or low (right) Mg levels in the nutrient solution. (i) Phosphorus deficiency in maize grown with optimal (left) or minimal (right) P levels in the nutrient solution.

phenylalanine ammonium lyase; PAL) and, importantly, late (e.g. dihydroflavonol 4-reductase (DFR); anthocyanidin synthase (ANS)) steps in the pathway. Moreover, genes responsible for anthocyanidin glycosylation and vacuolar sequestration are highly upregulated.

A complex hierarchical network of multiple transcription factors (TF) regulate the expression of genes encoding enzymes for anthocyanin synthesis (Zimmermann *et al*., 2004; Gonzalez *et al*., 2008; Tsay *et al*., 2011; Zhou *et al*., 2012). The transcriptional MBW complex (named after the TF families MYB, bHLH and WD40) activates the expression of several structural anthocyanin genes (Gonzalez *et al*., 2008; Tsay *et al*., 2011). This MBW-complex activity can be repressed by the SNF1-related protein kinase 1 (SnRK1), which also dissociates the MBW complex (Broucke *et al*., 2022). SNF1-related protein kinase 1 is known as a key mediator of energy signalling (Wurzinger *et al*., 2018), and it is suggested that this is a crucial strategy as anthocyanin biosynthesis is energy and carbon expensive, and its

tight regulation is needed to redistribute energy and carbon flow to more essential survival responses under extreme stress.

The TFs' production of anthocyanin pigment 2 (PAP2) and GLABRA3 (GL3) are of major importance for anthocyanin synthesis under nutrient depletion (Scheible *et al*., 2004; Bi *et al*., 2007; P. J. Lea *et al*., 2007; Peng *et al*., 2007a,b; Lillo *et al*., 2008; Feyissa *et al*., 2009). Besides inducing genes of the flavonoid pathway, GL3 also regulates the negative feedback response that stops anthocyanin production when the nutrient level is optimal (Nemie-Feyissa *et al*., 2014).

The effects of low temperature, high-light and UV irradiation on anthocyanin synthesis in vegetative tissues have been reviewed extensively (Steyn *et al*., 2002; Gould, 2004; Landi *et al*., 2015). In this review, a focus is put on foliar anthocyanins and (1) the diverse nutritional disorders that can cause anthocyanin accumulation in leaves and (2) nutrient-specific differences with respect to the anthocyanin localization and composition. There are physiological similarities between different nutrient stresses, and some metabolic

Table 1 List of anthocyanin responses in diverse plant species to various nutrient stresses.

Nutrient stress	Crop	Experimental conditions	Anthocyanin response	Reference
Nitrogen deficiency	Tomato (*Solanum lycopersicon*)	Low N supply (10–30 µM NO_3^-); nutrient film technique growing system	Anthocyanin accumulation in stems, leaves and roots	Larbat *et al.* (2012)
		No N supply; rockwool culture	Anthocyanin accumulation in leaves; increase negatively correlated with decreased temperatures; induction of MYB transcription factors	Lovdal *et al.* (2010)
	Apple	Tissue-cultured apple cells	Higher levels of anthocyanins in cells expressing the apple bZIP, HY5. Associated increases in nitrogen uptake	An *et al.* (2018)
	Cabbage (*Brassica oleracea* var. *capitata*), Cauliflower (*B. oleracea* var. *botrytis*), Kohlrabi (*B. oleracea* var. *gongylodes*), Radish (*Raphanus sativa*), Rapeseed (*Brassica napus*)	No N supply; hydroponics	Anthocyanin accumulation in hypocotyl of cabbage, cauliflower; in radish also in stem, petiole, midvein and in the lower epidermis of the cotyledons; localization in hypocotyl and petioles mainly in subepidermal layer; localization in cotyledons and leaf-blades located in epidermis and mesophyll cells; anthocyanoplasts prominent in petioles and cotyledons of cabbage	Hodges & Nozzolillo (1996)
	Arabidopsis thaliana pap1-D mutant, *5gt* mutant	Low N supply (10 mM NH_4NO_3, 9.4 mM KNO_3 (half-strength)); agar-solidified medium	Anthocyanin accumulation in leaf epidermis, light exposed mesophyll tissue, and in the vascular tissues in *pap1-D*; AVIs enriched in cyanidin-3-*O*-glucoside derivatives in *5gt*	Shi & Xie (2010); Chanoca *et al.* (2015)
	Arabidopsis thaliana pap1-D mutant	Various combinations and concentrations of NH_4^+ and NO_3^- salts in nutrient solution; agar-solidified medium	Anthocyanin concentration and molecular diversity increases with decreasing N supply; increased expression levels of *PAP1* and *TT8*, decreased expression of *LBD37*, *38* and *39*	Zhou *et al.* (2012)
Phosphorus deficiency	*Arabidopsis thaliana*	No P supply; seedling liquid culture	Increased total anthocyanin levels	Kovinich *et al.* (2014)
	Cabbage (*B. oleracea* var. *capitata*), Cauliflower (*B. oleracea* var. *botrytis*), Kohlrabi (*B. oleracea* var. *gongylodes*), Radish (*R. sativa*), Rapeseed (*B. napus*)	No P supply; hydroponics	Anthocyanin accumulation in hypocotyl of cabbage and cauliflower; in radish also in stem, petiole, midvein and in the lower epidermis of the cotyledons; only little anthocyanin accumulation in anthocyanoplasts	Hodges & Nozzolillo (1996)
Magnesium deficiency	Rice (*Oryza sativa*)	No Mg supply; hydroponics	Anthocyanin accumulation in (middle) leaves, accompanied by increased starch content and decreased chlorophyll concentration	Kobayashi *et al.* (2013)
Sulphur deficiency	Rapeseed (*B. napus*)	Low S supply (8.7 µM $MgSO_4$); hydroponics	Anthocyanin accumulation in young leaves, accompanied by an increase in H_2O_2 in the young leaves, increased intercellular CO_2 and decreased photosynthetic activity	D'Hooghe *et al.* (2013)
	Grapevine (*Vitis vinifera*)	No S supply; plantlet (*in vitro*) culture and cell (callus) culture	Moderate anthocyanin accumulation in plantlets, but not in cell cultures; upregulation of CHS, DFR, and ANS in plantlets; downregulation of CHS in cell cultures	Tavares *et al.* (2013)
Boron deficiency	Turnip (*Brassica rapa* var. *rapa*)	Low B supply (< 2.5 µM); hydroponics	Anthocyanin accumulation in leaves	Hajiboland & Farhanghi (2010)
Copper deficiency	Common bean (*Phaseolus vulgaris*)	Low Cu supply (1 µM $CuSO_4$); hydroponics	Anthocyanin accumulation in the crown of roots	Naya *et al.* (2014)

ANS, anthocyanidin synthase; AVI, anthocyanic vacuolar inclusions; bZIP, basic region/leucine zipper; CHS, chalcone synthase; CO_2, carbon dioxide; $CuSO_4$, copper sulphate; DFR, dihydroflavonol reductase; H_2O_2, hydrogen peroxide; HY5, ELONGATED HYPOCOTYL 5; KNO_3, potassium nitrate; LBD37, 38 and 39, Lateral Organ Boundary Domain 37, 38 and 39; $MgSO_4$, magnesium sulphate; NH_4^+, ammonium; NH_4NO_3, ammonium nitrate; NO_3^-, nitrate.

denominators are common nodes connecting various nutrient disorders with anthocyanin synthesis. The underlying genetics of foliar anthocyanin accumulation in response to specific plant nutrients has been studied in Arabidopsis. Such knowledge may allow for more precise identification of nutritional disorders in field-grown crops. This could be exploited for the technical advancement of nondestructive optical devices for detecting specific nutrient stress and thereby enable demand-oriented use of fertilizers. This is urgently needed regarding persistently high groundwater nitrate levels in many countries as a result of inappropriate N input from agriculture, with harmful consequences for human and environmental health, as well as biodiversity (Zhang *et al.*, 2015).

It is also important to note that nutrient disorders affect anthocyanin levels in fruits, and the relationship between anthocyanin content in fruits and the plant nutritional status has previously been reviewed (Jezek *et al.*, 2018). Their levels can either positively or negatively impact the fruit quality and market value, depending on consumer preferences. In this review, we will focus on anthocyanins in leaves that are increased temporarily under nutrient stress and whose ecophysiological functions differ from fruit anthocyanins, that are constitutively expressed to attract pollinators and seed dispersal agents.

Distinct patterns in foliar anthocyanins induced by different nutrient deficiencies

Nitrogen deficiency

Leaf reddening or purpling is a well-known symptom of N deficiency and prevalent, especially in species that are naturally high in anthocyanins such as *Brassica* (Fig. 1c), fruiting trees and berry bushes (Bergmann, 1993; Hodges & Nozzolillo, 1996), but can also occur in other species (Table 1). Blades, veins and petioles of older leaves turn purple first, because N is translocated out of this tissue into younger leaves. Concomitant chlorophyll breakdown is common (Fig. 1c–i) but not a prerequisite, since the genetic upregulation of anthocyanin-related genes has also been measured in stay-green cultivars of rapeseed (*Brassica napus*) under N starvation (Koeslin-Findeklee *et al.*, 2015). Nevertheless, it was recently indicated that chlorophyll breakdown (another process correlated with chlorophyll resorption), rather than anthocyanin synthesis, determines N translocation efficiency in trees (Pena-Novas & Archetti, 2021). QTL mapping of N-deficient plants suggests that anthocyanin synthesis and leaf yellowing are two genetically separate pathways, and plants respond to N limitation by promoting both processes independently (Diaz *et al.*, 2006).

Most often anthocyanin accumulation is not uniformly distributed across the leaf. In N-deficient Arabidopsis leaves, anthocyanins accumulate predominantly in the epidermis or subepidermal layers (Shi & Xie, 2010), but can spread throughout the mesophyll under prolonged deficiency (Hodges & Nozzolillo, 1996). Anthocyanins accumulate mainly in so-called anthocyanoplasts or anthocyanic vacuolar inclusions (AVIs) in response to N deficiency (Hodges & Nozzolillo, 1996; Liang & He, 2018; Socquet-Juglard *et al.*, 2020). The relationship of

anthocyanoplasts to AVIs is the subject of debate (Kallam *et al.*, 2017), but it appears that both of these subvacuolar pigment bodies may sequester specific anthocyanin species. For example, AVIs mainly contain aromatically acylated anthocyanins (Kallam *et al.*, 2017), as shown for example in grapevine (*Vitis vinifera*) cell culture (Conn *et al.*, 2003), and their formation is promoted by an increase in decorated cyanidin 3-*O*-glucoside derivatives in Arabidopsis (Pourcel *et al.*, 2010; Chanoca *et al.*, 2015). N deficiency can increase the molecular diversity of the anthocyanin profile (Shi & Xie, 2010; Zhou *et al.*, 2012), which might differ from P deficiency-induced anthocyanins (to be described later) where less AVI formation was observed although similar total anthocyanin amounts were detected (Hodges & Nozzolillo, 1996).

Notably, the absence of stress-induced anthocyanins does not necessarily reduce plant growth under N deficiency. Anthocyanin-less, (acyanic) Arabidopsis *transparent testa*-mutants that lack components of the anthocyanin synthesis pathway accumulate comparable or even slightly more biomass than the wild-type under N deficiency (Misyura *et al.*, 2013). Likewise, the capacity to remobilize nutrients from chlorophyll breakdown was not impaired in the acyanic plants supporting the findings of Pena-Novas & Archetti (2021) mentioned above. However, these experiments were performed under optimal growth conditions in terms of light intensity, temperature and humidity. It is to be expected that nutrient stress-induced anthocyanins do fulfil essential protective functions under more challenging environmental conditions. For example, in mutants defective in either the MYB activator of anthocyanin (MYB75 or PAP1), or the biosynthetic enzyme DFR, there was lower survival rate under very low N (Liang & He, 2018). These findings have been ascertained in the model plant Arabidopsis. An upregulation of several key enzymes and TFs of the flavonoid metabolism has also been documented in tomato and rapeseed leaves in response to N deficiency (Bongue-Bartelsman & Phillips, 1995; Lovdal *et al.*, 2010; Koeslin-Findeklee *et al.*, 2015).

New research is beginning to link the transcriptional cascade that switches on anthocyanin-related genes with nitrogen levels. The ER membrane-localized protein ROOT HAIR DEFECTIVE3 represses N deprivation-induced anthocyanins (Wang *et al.*, 2015). Anthocyanin formation is downregulated by TFs from the LATERAL ORGAN BOUNDARY DOMAIN (LBD) family under optimal N nutrition (Rubin *et al.*, 2009; Zhou *et al.*, 2012). The BTB gene family, short-lived proteins that are targeted for degradation by the 26S proteosome pathway, acts as conserved negative regulators of nitrate uptake genes (Araus *et al.*, 2016). In apple, BT2 targets the MYB activator of anthocyanins for degradation by COP1 (Kang *et al.*, 2022). As BT2 is induced by nitrate, a model is proposed (X. F. Wang *et al.*, 2018) whereby nitrogen deficiency releases MYB1 from repression, and therefore, anthocyanin levels are elevated by more abundant levels of the MYB activator. Recently, it was shown that nitrate deficiency induces expression of the 14-3-3 protein GRF11, which negatively interacts with (i.e. destabilizes) BT2 via 26S proteasome pathway increasing MYB1 abundance leading to expression of genes involved in anthocyanin biosynthesis (Ren *et al.*, 2021). Peng *et al.* (2007b) found that the ubiquitin ligase

nitrogen limitation adaptation (NLA) is essential in the specific reaction to N deficiency. Nitrogen-deprived nla mutants do not synthesize anthocyanins, but channel the metabolic flux towards lignin synthesis instead (Peng *et al.*, 2007a,b, 2008). Interestingly, anthocyanin synthesis induced by P deficiency is not impaired in nla mutants.

The gaseous hormone ethylene is also involved; nitrogen starvation-induced anthocyanin accumulation is enhanced in ethylene-insensitive Arabidopsis mutants (Ma *et al.*, 2022), but inhibited by treatment with the ethylene inducer ACC (Wang *et al.*, 2015). Gibberellic acid (GA) is also involved in integrating N-induced anthocyanin increases, via direct binding of the GA-DELLA protein to PAP1 (Zhang *et al.*, 2017).

Phosphorus deficiency

Besides N deficiency, phosphorus (P) deficiency is also an elicitor for nutrient stress-induced anthocyanin accumulation (Table 1). The stems, petioles, leaf bases and leaf veins turn red or purple first under P deficiency (Fig. 1i). However, red colouration can also spread across the leaf blade (Fig. 1f,h). In contrast to N deficiency, where anthocyanin accumulation is usually accompanied by chlorosis, P starvation often causes increased chlorophyll density per leaf area, and the leaves appear dark blueish-green. It has also been observed that the reddening emerges between the leaf veins under P deficiency, but along the veins under N deprivation (Barker & Pilbeam, 2007).

Cell-type- and tissue-specific accumulation of anthocyanins has been found in P-deficient C_4 species (Henry *et al.*, 2012; Hernandez & Munne-Bosch, 2015), but these findings remain species-specific and can therefore not be generalized. In contrast to N limitation, only minor accumulation of anthocyanins in vacuolar anthocyanoplasts was found in P-starved *Brassica* species (Hodges & Nozzolillo, 1996), which suggests that different anthocyanin species are synthesized under N and P deficiencies. Similar to the aforementioned results in N-deficient acyanic Arabidopsis mutants, no significant differences in the photosynthetic efficiency or growth were found in genetically similar maize and coleus varieties with contrasting anthocyanin content (Henry *et al.*, 2012). However, these findings do not rule out a significant adaptive function of anthocyanins in nutrient-stressed plants in the field. In wheat, varieties that accumulate anthocyanins perform better in response to P deficiency at the seedling stage (Kaur *et al.*, 2022). The authors indicated that very dark varieties have the most diverse profile of anthocyanins (including derivatives of cyanidin, delphinidin and peonidin) and show best adaptation to P stress.

Negative feedback regulation of anthocyanin formation has been reported for P deficiency (Chen *et al.*, 2007). In apple, P deficiency induces both the micro-RNA miR399 and the phosphate transporter PHT1;4, which then affects a MAPK kinase pathway to regulate the activity of MYB10, the key TF in anthocyanin biosynthesis (Peng *et al.*, 2020). SPX4, a negative regulator of P starvation-induced genes, has recently been found to bind the MYB TF PAP1 to repress *DFR* gene expression and anthocyanin biosynthesis (He *et al.*, 2021). The authors demonstrated that the SPX4/PAP1 interaction is diminished under low P conditions and

PAP1 is released from SPX4 repression. These findings illustrate that anthocyanin accumulation is precisely controlled as part of the physiological adjustment to nutrient stress.

The phytohormone abscisic acid (ABA) is implicated in the elevation of anthocyanin levels in response to low P. Lei *et al.* (2022) used ABA-insensitive Arabidopsis mutants to show that *abi1/abi2/abi3/abi5* mutants accumulated less anthocyanin than WT, while an *abi4* mutant showed greater accumulation, under low P. ABI5 is a key hub-TF, integrating light signals with HY5 to allow grow-not-grow decisions (Wang *et al.*, 2021). Perhaps this is one point where ABA levels and anthocyanin induction are interconnected, within a transcriptional cascade involving ABI5 and HY5.

Magnesium deficiency

The first visual symptom of magnesium (Mg) limitation is chlorosis, especially between veins of mature leaves. Accompanying leaf purpling has been reported for diverse species such as maize (*Zea mays*) (Fig. 1h) and rice (*Oryza sativa*) among others (Table 1). In rice seedlings, an increase in anthocyanin was observed only in older leaves (i.e. L4 and L5; Kobayashi *et al.*, 2013). Magnesium deficiency-induced anthocyanin accumulation usually occurs in older leaves, starts at the leaf margins and is most often preceded or accompanied by chlorophyll breakdown (Fig. 1f,h). This gradual appearance coincides with the successive Mg depletion of the leaf tissue starting from the leaf margins. By contrast, *Citrus sinensis* seedlings experiencing Mg deficiency showed decreased anthocyanin concentrations with the lowest concentrations in the older leaves (Li *et al.*, 2017). This might indicate that the anthocyanin response to Mg deficiency is species-specific or depends on the developmental stage in some species.

Sulphur deficiency

In some species, sulphur (S) deficiency has been shown to induce leaf purpling (Fig. 1e; Bergmann, 1993; D'Hooghe *et al.*, 2013). However, in contrast to N and Mg limitation, S deficiency-induced anthocyanin formation first appears in younger leaves (Bergmann, 1993; D'Hooghe *et al.*, 2013), because of the low re-translocation of S within the plant body. S deficiency-induced anthocyanin synthesis has been observed in *V. vinifera* (Tavares *et al.*, 2013). Several genes of the anthocyanin synthesis pathway were upregulated, and anthocyanins accumulated in S-deficient *V. vinifera* plantlets, but these findings could not be confirmed in cell cultures. Large anthocyanin increases were found in Arabidopsis in response to S deficiency (Robe *et al.*, 2020), whereas very small increases in the anthocyanin concentration were detected in S-deficient rice (Lunde *et al.*, 2008). The discrepancy in these results could be caused by species-specific differences, experimental setup and/or a more complex regulation where carbon-to-nitrogen balance is involved. Although leaf purpling can occur under S deficiency, it is not a general symptom.

During sulphur deficiency as key TF, SLIM1 is induced in a sugar-dependent manner (Wawrzyńska *et al.*, 2022). SLIM1 belongs to a small family of plant-specific TFs, which include EIN3 and EIL1, that regulate the ethylene-responsive gene
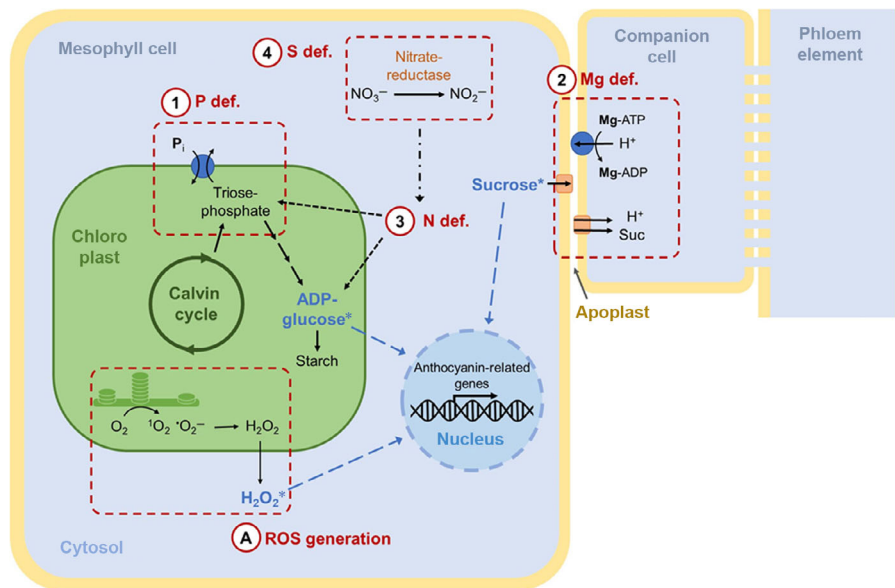
Fig. 2 Reactive oxygen species (ROS) and sugars as signalling entities in the pathway of anthocyanin gene induction. Potential metabolites with signalling function are marked with an asterisk (*). (A) Chloroplasts are a dominant source of ROS evolution under nutrient deficiency. Over-reduction in the electron transport chain leads to the generation of reactive compounds such as superoxide ($^{•}O_2^{-}$), which are further transformed into the more stable H_2O_2 which enters the cytosol. Sugar accumulation results but varies among different nutrient deficiencies. (1) Phosphorous (P) deficiency: Triosephosphate synthesized in the Calvin–Benson cycle is exported from the chloroplast in exchange for cytosolic inorganic P (P_i) via the triosephosphate/P_i translocator (TPT). The TPT activity is impaired under P_i deficiency, which leads to a built-up of photosynthesis intermediaries and ultimately starch synthesis (Hernandez & Munne-Bosch, 2015). (2) Magnesium (Mg) deficiency: Sucrose is exported from photosynthetically active tissue to the apoplast, from where it is loaded into phloem companion cells in symport with H^+. This thermodynamically active process depends on H^+ pumps in the plasma membrane energized by Mg-ATP hydrolysis. Mg deficiency decreases the H^+-ATPase activity and thus impairs the apoplastic phloem loading, leading to accumulation of photosynthates in mesophyll cells surrounding the phloem (Cakmak *et al.*, 1994a; Hermans & Verbruggen, 2005). (3) Nitrogen (N) deficiency: Sugar and starch accumulation is frequently observed under N deficiency (Hermans *et al.*, 2006). In Arabidopsis carbohydrate metabolism-related genes are differentially regulated under N starvation. Starch degradation is repressed whereas genes required for the export of photosynthate and its conversion to sucrose are upregulated (Scheible *et al.*, 2004). (4) Sulphur (S) deficiency: Sugar accumulation under S deficiency is presumably a secondary effect. S deficiency decreases the activity of the cytosolic nitrate reductase (Migge *et al.*, 2000). This inhibition results in physiological N deficiency with the same consequences described in (3).

expression. SLIM1 affects the expression of the R2R3 MYB PAP1 by directly binding to its promoter.

Other minerals

Anthocyanins were also reported to accumulate during the deficiencies of micronutrients. For example, *Brassica* and *Trifolium* species and red salad varieties showed red-purple leaf phenotypes upon boron deficiency (Bergmann, 1993; Hajiboland & Farhanghi, 2010). Copper deficiency lead to anthocyanin accumulation in roots of common bean (*Phaseolus vulgaris*; Naya *et al.*, 2014), and Rahimi & Bussler (1975) showed evidence of red leaf colouration in zinc-deficient maize plants. However, the underlying genetic regulation of micronutrient deficiency-induced anthocyanin biosynthesis has not been reported.

The role of anthocyanins in the acclimation to nutrient stress

Since their first scientific description and naming in 1835, the physiological function of leaf anthocyanins in nutrient-stressed plants has generated debate (Lillo *et al.*, 2008). One potential function involves sugar responses. As nutrient deficiency causes growth inhibition, this will reduce the sink strength for

photoassimilates and thus impacts sugar distribution within the plant (Fig. 2). In turn, photosynthesis will then be affected and dysfunctional chloroplasts will fail to export carbon assimilate (Araguirang & Richter, 2022). As anthocyanins can be highly decorated with sugar moieties (Zhao *et al.*, 2014), they may represent a carbon sink for sugar sequestration (Lo Piccolo *et al.*, 2018).

Anthocyanin levels are positively correlated with soluble sugar concentrations under N and P deficiency (Khavari-Nejad *et al.*, 2009), and anthocyanin synthesis can also be induced by external sugar feeding (Kumar & Sharma, 1999; Teng *et al.*, 2005) or artificial disruption of phloem loading in source leaves (Jeannette *et al.*, 2000; Lloyd & Zakhleniuk, 2004). This is further supported by *in vitro* trials showing that sucrose upregulates anthocyanin-related enzymes and thus anthocyanin biosynthesis (Zhou *et al.*, 2020). The major regulating TFs for anthocyanin synthesis, such as PAP1 in Arabidopsis, respond positively to increased sucrose concentrations (Lloyd & Zakhleniuk, 2004; Teng *et al.*, 2005; Solfanelli *et al.*, 2006), indicating a direct link between nutrient deficiency-induced sugar accumulation and anthocyanin synthesis. Two protein kinase complexes, SnRK1 and TOR, appear to connect sugar levels with MYB activators of anthocyanin such as PAP1 (Li *et al.*, 2021). Sugar accumulation is also a common symptom of K deficiency (Cakmak *et al.*, 1994a; Hermans

et al., 2006). However, K deficiency is usually not accompanied by leaf purpling (Hodges & Nozzolillo, 1996) and anthocyanin increase (Tjhia *et al.*, 2018), although this has been reported (Lawanson *et al.*, 1972) and may depend on experimental conditions.

The hypothesis that anthocyanins act as carbon stores under nutrient stress is still under debate. Kumar & Sharma (1999) showed that anthocyanins that accumulate under nutrient deficiency are not readily degraded after amelioration of the stress, which would argue against a function as reversible carbon stores. However, their anthocyanin measurements did not determine the level of anthocyanin glycosylation. It is therefore possible that sugar moieties were hydrolysed from the anthocyanin backbone once the nutrient stress was relieved. When assessing the potential of anthocyanins to sequester sugars, the quantitative differences in anthocyanin production and sugar accumulation in leaves under nutrient deficiency need to be considered. Sugar levels increase by several milligram per gram fresh weight when plants grow under nutrient stress (Cakmak *et al.*, 1994b; de Groot *et al.*, 2003; Hermans *et al.*, 2004), whereas anthocyanin concentrations rise by only a few hundred microgram per gram fresh weight in nutrient deficient leaves (Juszczuk *et al.*, 2004; Lovdal *et al.*, 2010; Larbat *et al.*, 2012). Based on a C balance calculation, Henry *et al.* (2012) considered anthocyanins not to be significant C sinks under P deficiency. However, the extent to which anthocyanins can contribute to sugar sequestration in nutrient-deprived plants has, to our knowledge, not been experimentally addressed in a systematic and detailed way and stronger experimental evidence is needed to answer this question.

Anthocyanins increase disproportionately to other flavonoids in N-stressed tomato leaves (Bongue-Bartelsman & Phillips, 1995), which indicates a more specific adaptive role of anthocyanins. Another major postulated function of nutrient-induced anthocyanins centres around reacting to, or ameliorating, free radicals and reactive oxygen species (ROS). Some anthocyanins have antioxidant activity and can scavenge ROS, that is they can donate an electron to a radical (Gould *et al.*, 2002; Kytridis & Manetas, 2006). On the contrary, the epidermal accumulation of anthocyanins enhances photon shielding of mesophyll chloroplasts, thus reduces the formation of ROS during photosynthetic light reaction under conditions of excessive irradiation. As nutrient deficiency often impairs metabolic pathways downstream of the photosynthetic light reaction, deficiencies will likely lead to over-reduction in final acceptors of chloroplast electron transport chain. This may cause ROS production when the chloroplast is impaired by nutrient starvation and reduces the capacity for energy utilization. Anthocyanins have the potential to mitigate damage in the chloroplast by absorbing excess light quanta and shielding chloroplasts, preventing ROS formation and by scavenging ROS that accumulates as a result of the electron transport chain not being able to pass electrons onto NAD(P)H. As mentioned before, anthocyanins are mainly localized in the epidermal layers where they can effectively function as light shields (i.e. photon shielding of mesophyll chloroplasts). Their maximal light absorption lies in the green region, but they also intercept photons that would otherwise be absorbed by chlorophyll (Gould, 2004; Nichelmann, 2014).
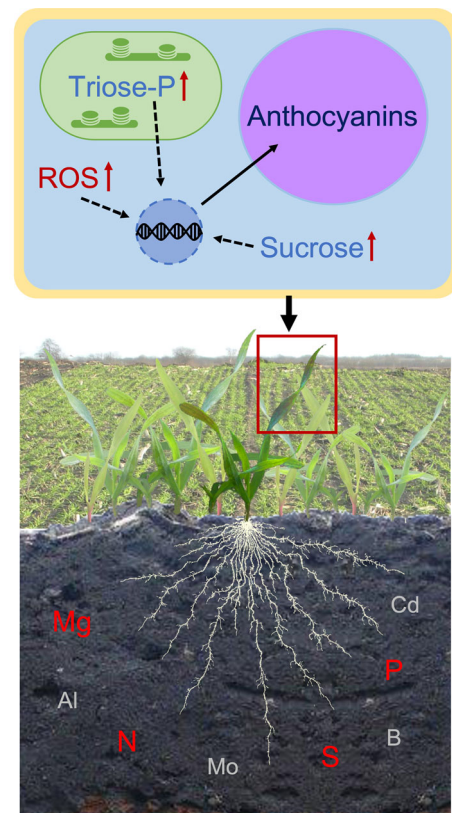
Fig. 3 Schematic overview of nutrient stress-induced anthocyanin synthesis. A diverse array of nutrient deficiencies (red) and nutrient toxicities (white) in the soil can cause foliar anthocyanin accumulation. Common metabolic nodes in the signalling pathway for genetic upregulation of anthocyanin synthesis may be increased sugar levels in the chloroplast and/or cytosol in addition to production of stress-related reactive oxygen species (ROS). Al, aluminium; B, boron; Cd, cadmium; Mg, magnesium; Mo, molybdenum; N, nitrogen; P, phosphorus; S, sulphur.

The finding that sufficient light intensity is a prerequisite for N deficiency-induced anthocyanin synthesis provides some evidence for a light-attenuating function under nutrient limitation (U. S. Lea *et al.*, 2007; Shi & Xie, 2010). Also, anthocyanin-containing leaves can have a reduced pool of xanthophyll-cycle components, indicating reduced nonphotochemical quenching (NPQ) (Manetas *et al.*, 2002; Kytridis *et al.*, 2008) and necessitating an alternative photoprotective means.

The photoprotective function of anthocyanins has been described for autumnal purpling of trees preceding leaf abscission (Kytridis *et al.*, 2008; Nikiforou *et al.*, 2011). These studies suggest that photoprotective anthocyanins might extend the lifespan of senescing leaves and enable extended nutrient retrieval, which is also advantageous under nutrient deficiency. However, no effect on the senescence process or the capacity to remobilize nutrients could be found in acyanic Arabidopsis mutants under N deficiency (Misyura *et al.*, 2013). It is therefore questionable if light attenuation by anthocyanins is essential for controlled chlorophyll breakdown and efficient nutrient retrieval (Koeslin-Findeklee *et al.*, 2015). As described above, anthocyanins accumulate in combination with chlorophyll breakdown (e.g. under N and Mg deficiency), but also under P deficiency that leads to increased

chlorophyll density. No consistent evidence for a photosynthetic advantage of anthocyanin production could be found in P-deficient maize (Henry *et al.*, 2012).

Recently, it was proposed that the antioxidant capacity of anthocyanins is of higher importance for photoprotection under short-term high-light exposure than the anthocyanin photon shielding effect (Yu *et al.*, 2021). This bioactivity is certainly relevant to nutrient deficiencies affecting photosynthesis, but also many other nutrient deficiency-induced dysfunctions. Increased anthocyanin accumulation and enhanced peroxidase activity were interpreted as an adaptation to ROS stress in P-starved tomato leaves (Khavari-Nejad *et al.*, 2009). However, as anthocyanins mainly accumulate in the vacuole of outer leaf layers, they are not optimally localized to scavenge short-lived ROS, (e.g. singlet oxygen or hydroxyl radicals), which are primarily produced in chloroplasts and mitochondria (Nichelmann, 2014) under nutrient deficiency. However, hydrogen peroxide (H_2O_2), which accumulates under nutrient stress, has a wider radius of damage. H_2O_2 enters the vacuole either by passive diffusion or through tonoplast intrinsic proteins (Bienert *et al.*, 2006; Agati *et al.*, 2013). Hence, a vacuolar scavenging system could be important to increase the ROS detoxification capacity (Yamasaki, 1997; Gould *et al.*, 2002; Bienert *et al.*, 2006) under nutrient stress. This 'secondary antioxidant system' comprises vacuolar peroxidases that reduce entering H_2O_2 by using flavonoids such as anthocyanins as electron donors (Bienert *et al.*, 2006); the flavonoid radicals are then reduced (i.e. recycled) by ascorbate (Agati *et al.*, 2013). It was shown that anthocyanins can scavenge H_2O_2 in the presence of a peroxidase *in vitro* (Yamasaki, 1997). Comparisons between the H_2O_2 scavenging efficiency of cyanic and acyanic leaf mesophyll cells indicate that this also occurs *in planta* (Gould *et al.*, 2002). Furthermore, anthocyanins in the vacuole are acted upon by vacuolar peroxidases (Zipor *et al.*, 2015).

Anthocyanin accumulation was observed in response to elevated H_2O_2 levels in Arabidopsis seedlings with simultaneous upregulation of PAP1 and TT8, two key transcriptional regulators of anthocyanin biosynthesis, as well as MYB113 and MYB114 (Xu *et al.*, 2017). Acyanic Arabidopsis mutants upregulate genes that are involved in ROS scavenging under N deficiency (Peng *et al.*, 2007b), indicating that alternative detoxification mechanisms are activated to compensate for the lack in anthocyanins. By performing RNA-Seq of maize roots, previously starved of N, then exposed to nitrate and ammonium, distinct transcriptional responses were observed (Ravazzolo *et al.*, 2020). Both N sources repress genes related to ROS detoxification. Ammonium induces the accumulation of foliar anthocyanins, while reducing overall total foliar flavonoid accumulation.

Why then do plants blush when they are hungry?

Anthocyanins are more than an efficient energy outlet. Sugar accumulation and/or oxidative stress likely act as signalling entities that induce anthocyanin synthesis under nutrient stress (Fig. 3). In combination with other nutrient-specific physiological responses, these triggers may lead to the fine-tuned generation of stress-specific anthocyanin patterns. Detailed spatiotemporal analysis of the anthocyanin profiles formed under different nutrient disorders could help answer Outstanding questions about their signalling and synthesis pathway as well as their specific physiological function. As discussed, it is likely that the antioxidant and light-shielding properties of anthocyanins confer the strongest adaptive advantage when under nutrient stress, whereas their functionality as reversible carbon stores needs stronger experimental evidence but may be of lesser physiological significance. Their specific localization in the epidermal leaf layers (often abaxial and sometimes adaxial) corroborates this assertion. Recent results suggest that accumulated sugars and ROS still play substantial roles as signalling entities. Both are physiological denominators that several nutrient stresses have in common, and they might represent metabolic nodes in the signalling pathway of anthocyanin synthesis (Fig. 2). Sugar signalling has also been shown to play a vital role in anthocyanin induction under high-light stress (Zirngibl *et al.*, 2023) and the role of sugar signalling in anthocyanin synthesis in general was reviewed by Van den Ende & El-Esawe (2014). Sugar-induced anthocyanin accumulation has also been shown in fruits (Zheng *et al.*, 2009; Dai *et al.*, 2014; Hu *et al.*, 2016; Liu *et al.*, 2017), and some underlying molecular regulators have been found, such as the hexokinase MdHXK1 (Hu *et al.*, 2016) and the Ser/Thr protein kinase MdSnRK1.1 (Liu *et al.*, 2017) in apple. The regulatory mechanisms that integrate cellular sugar and nutrient levels and dovetail these with anthocyanin synthesis, however, remain to be identified.

Also, differences can exist in the transcriptional regulation of sugar- or nutrient deficiency-induced anthocyanin synthesis, respectively. For example, the sucrose-induced anthocyanin synthesis pathway is controlled by the R2R3 MYB TF PAP1 (Lloyd & Zakhleniuk, 2004; Teng *et al.*, 2005; Solfanelli *et al.*, 2006; Peng *et al.*, 2008), whereas under N deficiency the close homologue PAP2 appears responsible for at least part of the anthocyanin response (Peng *et al.*, 2007a,b; U. S. Lea *et al.*, 2007). Therefore, increasing sugar levels *per se* are presumably not the sole elicitors for nutrient stress-induced anthocyanin synthesis. Suboptimal nutrition is also associated with changes in cellular proton and ion activities. Of note, pH and ionic status affect anthocyanin profile by determining the chemical reactivity of the pigments (Kallam *et al.*, 2017). Intriguingly, sugar and ROS accumulation under nutrient stress is likely highest in the most photosynthetically active tissue, that is palisade cells, while anthocyanins predominantly increase in epidermal layers where photosynthetic rate is likely less. Conceivably anthocyanin induction could be between cell layers, with evidence suggesting ABA (Trull *et al.*, 1997), GA (Jiang *et al.*, 2007), cytokinin (Y. Wang *et al.*, 2018), jasmonic acid (An *et al.*, 2021) and ethylene (El-Kereamy *et al.*, 2003) are all involved in an anthocyanic response.

Elevation of foliar anthocyanins is not a specific symptom for nitrogen or phosphorous deficiency but can be caused by a multitude of other nutritional disorders. There are specificities in anthocyanin distribution and profile among different nutrients. Thus, the potential of using anthocyanins as bioindicators for demand-oriented nutrient application can be exploited. We emphasize that foliar purpling/reddening have the potential to be used for diagnosing nutrient stress in green plants. Organ- and tissue-specific differences between individual nutrient deficiencies

exist, but the understanding of this is so far too scarce to use such patterns as diagnostic bioindicators. Also, the current noninvasive optical sensors for assessing foliar compounds such as chlorophyll and flavonoids are not precise enough to reliably distinguish between nutritional disorders (Kalaji *et al.*, 2017, 2018). Future research that focusses on the Outstanding questions can exploit the potential of anthocyanins as bioindicators for demand-oriented fertilizer application.

Outstanding questions

Which anthocyanin species are formed in which concentrations and cells under what nutrient conditions? What is the micro-distribution of nutrient-elements in different nutrient-deficient tissues?

How do sugars and ROS integrate signalling pathways of nutrient deficiency-caused anthocyanin synthesis? How is the information perceived and transmitted? Is systemic signalling between different tissue types involved?

Are nutrient-specific cellular changes such as cytosolic and vacuolar pH or ionic changes determining the anthocyanin profile?

Which anthocyanins are most effective in light absorption or radical scavenging?

To what extent can anthocyanins serve as reversible carbon storage compounds to 'buffer' increasing cellular sugar concentrations under nutrient deficiency?

Can foliar anthocyanins be reliably detected with noninvasive optical tools, for example based on chlorophyll fluorescence measurements? Do these measurements reflect the nutritional status of the plant? Can they serve to differentiate between nutritional disorders?

What is the genetic background of stress-induced anthocyanin synthesis in plants other than the model organism Arabidopsis?

Can strategic application of fertilizers or breeding increase foliar anthocyanin levels to enhance stress resilience?

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Competing interests

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