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Synergetic effects of nitrogen depletion, temperature, and light on the content of phenolic compounds and gene expression in leaves of tomato

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ABSTRACT

Tomato plants (*Solanum lycopersicum*, cv. Suzanne) were subjected to complete nutrient solution or a solution without nitrogen (N), and placed at different temperatures and light conditions to test the effects of environment on flavonoids and caffeoyl derivatives and related gene expression. N depletion during 4–8 days resulted in enhanced levels of flavonoids and caffeoyl derivatives. Anthocyanins showed pronounced increased levels when lowering the growth temperature from 24 °C to 18 °C or 12 °C. Flavonol levels increased when the light intensity was increased from 100 μmol m^{-2} s^{-1} PAR to 200 μmol m^{-2} s^{-1} PAR. Synergistic effects of the various environmental factors were observed. The increase in content of quercetin derivatives in response to low temperatures was only found under conditions of N depletion, and especially at the higher light intensity. Expression of structural genes in the phenylpropanoid and flavonoid pathways, *PAL* (*phenylalanine ammonia lyase*), *CHS* (*chalcone synthase*), *F3H* (*flavanone 3-hydroxylase*), and *FLS* (*flavonol synthase*) increased in response to N depletion, in agreement with a corresponding increase in flavonoid and caffeoyl content. Expression of these structural genes generally also increased in response to lower temperatures. As indicated through expression studies and correlation analysis, effects of N depletion were apparently mediated through the overall regulators of the pathway the MYB transcription factor ANT1 (ANTHOCYANIN 1) and SlJAF13 (a bHLH transcription factor orthologue of petunia *JAF13* and maize *RED* genes). A *PAL* gene (*PAL6*) was identified, and correlation analysis was compatible with *PAL6* being an actively expressed gene with function in flavonoid synthesis.

1. Introduction

Flavonoids contribute to increased quality of fruit, vegetables, processed food and beverages, and have potential health benefits (Cheynier, 2006; Heim et al., 2002). Flavonoids are also important for plants to adapt to various environmental conditions (Gould and Lister, 2006). Flavonoids and other phenolic compounds absorb UV light, and plants able to synthesize these compounds were more tolerant to high UV irradiation than mutants impaired in the flavonoid pathway (Clé et al., 2008; Gould and Lister, 2006; Li et al., 1993). Although not well understood, work on *Arabidopsis thaliana* revealed that the flavonoid pathway was important for better tolerance to nitrogen (N) starvation (Peng et al., 2008). Flavonoids are also involved in the resistance to pathogens and in acting as feeding deterrents (Gould and Lister, 2006). A high level of flavonoids in the plant may lower the needs for pesticide treatments.

In tomato leaves, the content of phenolic compounds has been found to increase in response to N depletion (Bongue-Bartelsman and Phillips, 1995; Stewart et al., 2001; Stout et al., 1998), but phenolic accumulation in leaves does not seem to be tightly coupled to that in ripe fruit (Bénard et al., 2009; Bovy et al., 2002; Stewart et al., 2001), where the content of these compounds are scarce in comparison. Subdued light irradiance as compared to plants grown under standard greenhouse light conditions, significantly reduced the levels of chlorogenic acid and rutin (a quercetin derivative) content in tomato leaves (Wilkens et al., 1996), and also the levels of ascorbate, lycopene, β-carotene, rutin, and caffeoyl derivatives in tomato fruit (Gautier et al., 2008). N supply and irradiance interact strongly, and in a complex manner. For example, at low N supply, relative growth rate was higher at low irradiance, whereas the opposite was true at high N supply (de Groot et al., 2002). Different environmental factors will interact and influence flavonoid levels in plants (Lillo et al., 2008).

Little is known about the underlying mechanisms inducing the responses referred to above, and how gene expression affects the

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regulation of the flavonoid pathway in tomato (Bongue-Bartelsman and Phillips, 1995; Gautier et al., 2008; Liu et al., 2004). Thus, further studies to determine how nutrient status may be employed to promote leaves and fruit of tomato high in phenolic compounds are needed.

In all higher plants studied the flavonoid pathway have been found to be regulated by MYB and bHLH transcription factors (Ramsay and Glover, 2005). *A. thaliana* is especially well analyzed, and the MYB factors (PAP1, PAP2) and bHLH factors (EGL3, GL3, TT8) are known to form complexes, which are stabilized by a constitutively expressed WD40 protein (TTG1) (Lepiniec et al., 2006). Regulations of the MYB and bHLH transcription factors are subjected to tissue, developmental, and environmental factors. In *A. thaliana*, *PAP1*/*2* and *GL3* expression was strongly induced by N depletion (Feyissa et al., 2009; Lillo et al., 2008). Although not as detailed known as in *A. thaliana*, the flavonoid pathway in flowers of *Petunia hybrida*, a member of the Solanaceae like tomato, is also much studied (Quattrocchio et al., 1998, 2006). In petunia, the MYB factor AN2 interacts with two bHLH transcription factors AN1 and JAF13, and a WD40 protein (AN11) is also known to be involved in the control of petunia flower pigmentation (de Jong et al., 2004; Quattrocchio et al., 2006). In tomato, a MYB factor (ANT1) enhancing flavonoid synthesis was identified by activation tagging (Mathews et al., 2003), and gene mapping showed the presence of tomato orthologues of *JAF13*, *AN1* as well as *AN11* (de Jong et al., 2004; Quattrocchio et al., 1998). However, to date no EST has been deposited in the gene banks for *AN1*. The function and characterization of these regulators still needs to be explored in tomato.

At present (October, 2009), only 50% of the tomato genome is sequenced (http://sgn.cornell.edu/about/tomato_sequencing.pl). Thus, the identities of genes involved in the flavonoid/phenylpropanoid pathway are not firmly established. To overcome this problem, candidate structural genes and regulators of anthocyanin/phenolics biosynthesis in tomato have been mapped according to homologues in other Solanaceae, and Southern blot and dot blot analyses have been used to estimate their copy numbers (Chang et al., 2008; de Jong et al., 2004; Mathews et al., 2003). Based on these previous publications, we assayed the relative expression of genes involved in the anthocyanin/phenolics biosynthesis pathway of tomato leaves, by means of quantitative real-time PCR. In cases where multiple loci of a gene have been found, the gene reported to be most actively transcribed was analyzed (Fig. 1). In addition, we also analyzed the expression of a novel putative *phenylalanine ammonia lyase* (*PAL*) gene (*PAL6*), and a novel putative bHLH transcription factor (*SlJAF13*), both for which expression in leaves could be easily enhanced by altering growth conditions. The effects of different environmental parameters (N, temperature, and light) and their synergetic effects on accumulation of phenolics were investigated. Measurements of leaf content of anthocyanins and the predominant flavonols combined with expression analysis of structural genes *PAL5*, *PAL6*, *CHS2*, *F3H*, *F3'H*, *F3'5'H*, *FLS*, and *DFR* (*dihydroflavonol reductase*) and regulatory genes *ANT1* and *SlJAF13* in the flavonoid/phenylpropanoid pathway provide data that contribute to the understanding of the molecular basis for accumulation of the various compounds.

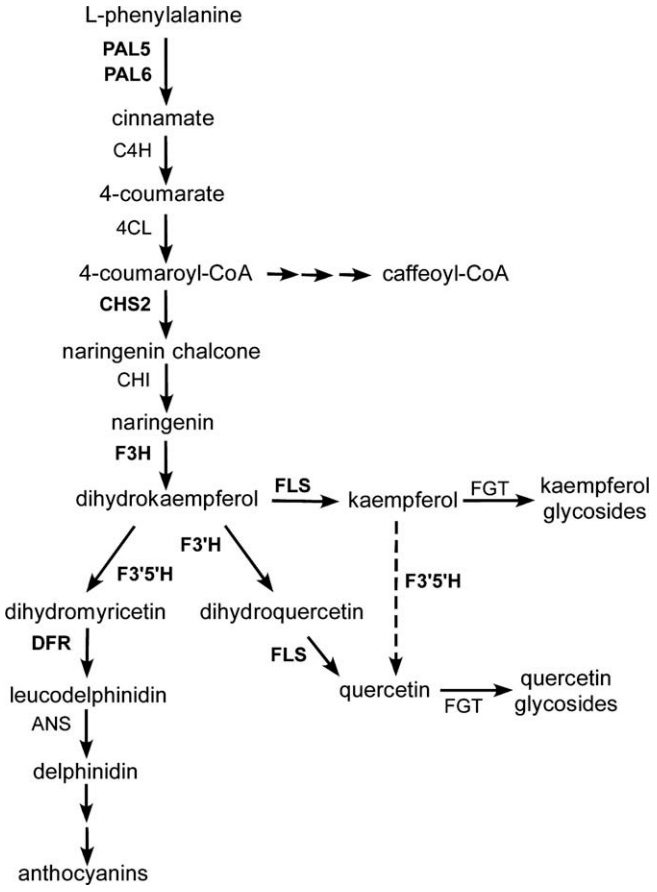

Fig. 1. Simplified scheme for flavonoid and phenylpropanoid synthesis in tomato. Structural genes analyzed are in bold print. The steps are catalyzed by phenylalanine ammonia lyase (PAL), for which multiple *PAL* genes encode, but *PAL5* is hypothesized to be the one most actively transcribed (Chang et al., 2008). At least three loci corresponding to chalcone synthase (CHS) are reported, but *CHS2* is believed to be the most important one (de Jong et al., 2004). Flavanone 3-hydroxylase (F3H), flavonol synthase (FLS), and dihydroflavonol 4-reductase (DFR) are reported to be encoded by single copy genes (de Jong et al., 2004). The copy number for flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) is presently not firmly established. F3′H has not been characterized from tomato, but is assumed to catalyze hydroxylation of dihydrokaempferol to dihydroquercetin. At least *in vitro* F3′5′H will hydroxylate dihydrokaempferol to give dihydroquercetin and dihydromyrecetin, dihydroquercetin to give dihydromyricetin, and kaempferol to give quercetin (Olsen et al., 2010). Other abbreviations: C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CHI, chalcone isomerase; ANS, anthocyanidin synthase; FGT, flavonol glycosyltransferase.

2. Results

2.1. Anthocyanins, flavonols, caffeoyl derivatives

The level of anthocyanins increased gradually as the temperature was lowered from 30 to 24, to 18 and 12 °C. Anthocyanin level was negatively correlated with temperature, irrespectively of the other environmental factors tested ($-0.63 < r < -0.86$, $p < 0.001$; Fig. 2A, Supplementary Table 1). There was a clear positive effect of N depletion which resulted in a further increase in anthocyanin levels (Fig. 2A, Table 1). Positive effect of the higher light intensity (200 μmol photons m^{-2} s^{-1} as compared to 100 μmol photons m^{-2} s^{-1} continuous white light) was not clear at all temperatures, but was significant at 18 °C under N depletion (Fig. 2A).

Accumulation of flavonols, i.e., various quercetin and kaempferol glycosides, was different from the accumulation of anthocyanins with respect to environmental factors. Quercetin 3-rutinoside (rutin) constituted in general ~90% (data not shown) of the total quercetin derivatives (w/w). Quercetin derivatives were negatively correlated to temperature, with the exception of plants given full nutrient solution for 8 days (Fig. 2B). A two- to fourfold increase in quercetin derivatives was observed when comparing plants grown for 8 days, high light treatment, and N depletion, to respective N replete plants. The lowest increase was in plants grown at 30 °C. Under N depletion, a clear positive effect of lowering the temperature was observed, especially after 8 days of treatment (Fig. 2B). The effect of N depletion on flavonols depends on temper-