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Modulation of chlorogenic acid biosynthesis in *Solanum lycopersicum*; consequences for phenolic accumulation and UV-tolerance

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ABSTRACT

Chlorogenic acid (CGA) is one of the most abundant phenolic compounds in tomato (*Solanum lycopersicum*). Hydroxycinnamoyl CoA quinate transferase (HQT) is the key enzyme catalysing CGA biosynthesis in tomato. We have studied the relationship between phenolic accumulation and UV-susceptibility in transgenic tomato plants with altered HQT expression. Overall, increased CGA accumulation was associated with increased UV-protection. However, the genetic manipulation of HQT expression also resulted in more complex alterations in the profiles of phenolics. Levels of rutin were relatively high in both HQT gene-silenced and HQT-overexpressing plants raised in plant growth tunnels. This suggests plasticity in the flux along different branches of phenylpropanoid metabolism and the existence of regulatory mechanisms that direct the flow of phenolic precursors in response to both metabolic parameters and environmental conditions. These changes in composition of the phenolic pool affected the relative levels of UV-tolerance. We conclude that the capability of the phenolic compounds to protect against potentially harmful UV radiation is determined both by the total levels of phenolics that accumulate in leaves as well as by the specific composition of the phenolic profile.

1. Introduction

Phenylpropanoid-derived compounds are ubiquitous plant secondary products (Albrecht et al., 1999; Winkel-Shirley, 2002; Grotewold, 2006). These compounds are involved in a broad range of physiological and ecological processes, ranging from recruitment of pollinators and seed dispersers, interactions with pathogens, nitrogen-fixing bacteria and parasitic roots in the rhizosphere, control of male fertility, UV-tolerance, antioxidant-based defence, auxin transport, and defence against microbes and grazers (Albrecht et al., 1999; Winkel-Shirley, 2002; Grotewold, 2006). This vast range of biological functions is matched by an equally vast structural diversity. It has been estimated that some 8000 different phenolic compounds are synthesised by plants (Albrecht et al., 1999), via a variety of polymerisation, hydroxylation, methylation, glycosylation, acylation, prenylation and condensation reactions (Pourcel et al., 2007).

The accumulation of phenolic compounds is a carefully controlled process with both the levels of phenolics and the composition of the phenolic pool varying considerably between organisms, tissues, developmental stages and in relation to environmental conditions (Winkel-Shirley, 2002). Expression levels of several genes encoding key enzymes in the early part of the biosynthetic pathway, called the general phenylpropanoid pathway, are elevated in response to UV-B radiation as well as a range of other environmental signals (Weisshaar and Jenkins, 1998; Long and Jenkins, 1998). These enzymes include phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate:coenzyme A ligase (4CL). PAL catalyses the conversion of phenylalanine to cinnamic acid, the first step of the general phenylpropanoid pathway, and this enzyme in particular controls the flux of precursors in to the phenol network and, as such, is critical in determining overall levels of phenolics that accumulate (Bate et al., 1994; Howles et al., 1996). Subsequent targeting of the phenolic precursors towards specific branches of the phenylpropanoid network is also controlled by biochemical, genetic, environmental and developmental parameters (Winkel-Shirley, 2002), and this involves the activity of a range of regulatory genes (Koes et al., 2005).

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Environmental factors like UV-B also impact on the activity of enzymes of the phenylpropanoid metabolism. For example, Wilson et al. (2001) showed that UV-B stimulates the hydroxylation reaction that converts quercetin in to kaempferol. It is likely that the allocation of phenolic substrates to individual branches of the phenylpropanoid pathway is also subject to metabolic regulation, including substrate and product feedback-loops. For example, in *Arabidopsis thaliana*, the *tt4* mutant lacks chalcone synthase (CHS) activity and consequently does not accumulate flavonoids. However, this mutant accumulates 30–60% more sinapoyl esters and this has been speculated to reflect shunting of 4-coumaroyl-coenzyme A from the blocked flavonoid pathway into sinapate biosynthesis (Li et al., 1993). The *Arabidopsis thaliana tt5* mutant also lacks flavonoids, and this is the result of impaired chalcone–flavanone isomerase activity (Li et al., 1993). However, the *tt5* mutant contains lowered levels of sinapoyl esters, and consequently, is considerably more UV-sensitive than the *tt4* mutant (Li et al., 1993; Booij-James et al., 2000). The lack of sinapoyl ester accumulation in the *tt5* mutant reflects regulatory interactions between flavonoid and sinapoyl ester biosynthesis (Li et al., 1993). In recent years our understanding of such regulatory interactions between the different branches of phenylpropanoid metabolism has been improved with the discovery of the R2R3 family of MYB transcriptional activators (Sablowski et al., 1994; Mathews et al., 2003; Verdonk et al., 2005) and repressors (Jin et al., 2000). Transcriptional de-repression, resulting from decreased expression of transcriptional repressors, appears to be one of the key mechanisms influencing flux through specific branches of the phenylpropanoid network, in response to UV-B light (Jin et al., 2000).

Phenolic compounds play a key role in protecting plants from potentially harmful UV-B radiation. In plants, UV-B has the potential to affect metabolic processes such as growth, morphogenesis, photosynthesis, and flowering. UV-B also catalyses the dimerisation of nucleotide bases which, in turn, has consequences for DNA transcription and replication (Hollosy, 2002; Jansen et al., 1998; Jordan, 1996). Numerous studies have demonstrated that UV exposure results in increases in the overall levels of phenolic compounds in response to UV exposure (Hollosy, 2002; Jansen et al., 1998; Jordan, 1996). Phenolics protect plants by specifically absorbing light of wavelengths between 280 and 340 nm, and therefore do not decrease the penetration of photosynthetically active radiation into the leaf, while filtering out harmful UV-B. Many phenolics are also effective antioxidants and this underpins the functional role of these compounds in a broad spectrum of plant stress responses. Substantial levels of phenolics accumulate in the upper epidermal cell layer, where they are mostly located in the vacuoles. As a result, little UV-B radiation (<10%) actually penetrates the mesophyll (Turunen et al., 1999), and the direct, growth-inhibiting effects of natural doses of UV-B are generally minor.

Different plant species accumulate different phenolics. The most abundant soluble phenolic in tomato leaves is chlorogenic acid (Niggeweg et al., 2004). The synthesis of CGA from quinic acid and caffeoyl CoA is catalysed by the enzyme hydroxycinnamoyl CoA quinate transferase (HQT; Niggeweg et al., 2004). We have investigated the relationship between accumulation of CGA, and other phenolic compounds, and UV-susceptibility in tomato plants (*Solanum lycopersicum* cv. Moneymaker) with altered HQT expression.

2. Results

Hydroxycinnamoyl CoA quinate transferase (HQT) is the key enzyme catalysing CGA biosynthesis in tomato. Transgenic tomato plants (*S. lycopersicum* cv. Moneymaker) that overexpress HQT (OE) or that are HQT gene-silenced (GS) have been generated and characterised by Northern blotting, as previously detailed (Niggeweg et al., 2004). Cuttings from original transformants were used in this study. Plants that were raised in growth rooms (indoors) were exposed to relatively low fluence rates of photosynthetically active radiation (PAR), and spectra that contained some UV-A (shortest wavelength 375 nm), but lacked UV-B (Fig. 1). Plants that were raised in a tunnel were exposed to relatively high, ambient light conditions, including natural background levels of UV-A and UV-B (Fig. 1). Indoor and tunnel-growth conditions differed also in respect to temperature profiles, relative humidity, and air circulation (wind).

We measured the accumulation of soluble phenolic compounds in methanol extracts. Overall, we found that the levels of soluble phenolics were relatively low in leaves of plants that were raised under growth room conditions compared to tunnel-grown plants (Fig. 2). Yet, there were significant differences between the four tomato lines indicating that alterations in HQT expression affected the overall accumulation of phenolic compounds under growth room conditions. Levels of UV absorbance were particularly low in gene-silenced plants, but significantly higher in WT plants raised in growth rooms (Fig. 2). The highest level of phenolic compounds was found in HQT-overexpressing plants. Tomato plants grown in a tunnel under natural light conditions were characterised by a major increase (up to 3.5-fold) in the overall level of UV-absorbing compounds compared to growth room-grown plants (Fig. 2). The difference between tunnel-grown and growth room-grown plants was particularly strong for the gene-silenced plants, whereas it was relatively small for the HQT-overexpressing plants. As a consequence, the relative differences in total phenolic accumulation between the four tomato lines were less pronounced under natural light conditions. We found that the HQT-overexpressing plants contained on average, some 55% more UV-absorbing pigments than gene-silenced plants under tunnel light conditions.

Chlorogenic acid is the most abundant phenolic compound in tomato leaves (Niggeweg et al., 2004). In order to determine whether altered HQT expression impacted the levels of CGA and other phenolic compounds under the growth conditions we used, we analysed the composition of the soluble phenolic pool. Methanolic extracts of leaves of tomato plants contained several UV-absorbing compounds. Peaks in the UV chromatogram were identified using mass spectrometry. The major peaks in the LC–MS spectrum represented CGA (peaks B, D, E and G) (Fig. 3). The identity of these peaks was confirmed using mass spectrometry (Fig. 4). Similar mass spectra are expected for different chlorogenic acid isomers, several of which are known to be present in tomato including 3-caffeoyl quinate (neochlorogenic acid), 4-caffeoyl quinate (cryptochlorogenic acid) and 5-caffeoyl quinate (Niggeweg et al., 2004). Also present in the spectrum were rutin (peak J) and rutin–pentose (peak I), quercetin–glucose (peak K), kaempferol–rhamnose–glucose (peak L), tomatine (peak N) and dehydrotomatine (peak M). CGA made up some 67% of total, soluble UV-absorbing material of wild type leaves raised under growth room conditions. In comparison, rutin (peaks I and J) the second most abundant phenolic in tomato leaves comprised less than 13% of total UV-absorbing capacity (Table 1). The growth-room-raised, gene-silenced lines accumulated nearly 70% less CGA than wild type plants and this was reflected in a drop in total soluble phenolics (Fig. 2). HQT-overexpressing lines, raised under growth room conditions, contained substantial amounts of CGA which comprised, on average, some 80% of the absorbance present in methanolic extracts. Compared to the wild type, HQT-overexpressing plants accumulated nearly 2.5-fold more CGA. Rutin and rutin–pentose (peaks I and J), flavonoids (peaks K and L) and tomatine levels (peaks M and N) varied little between the growth room-grown lines (Table 1). Thus, the shift in phenolic profiles

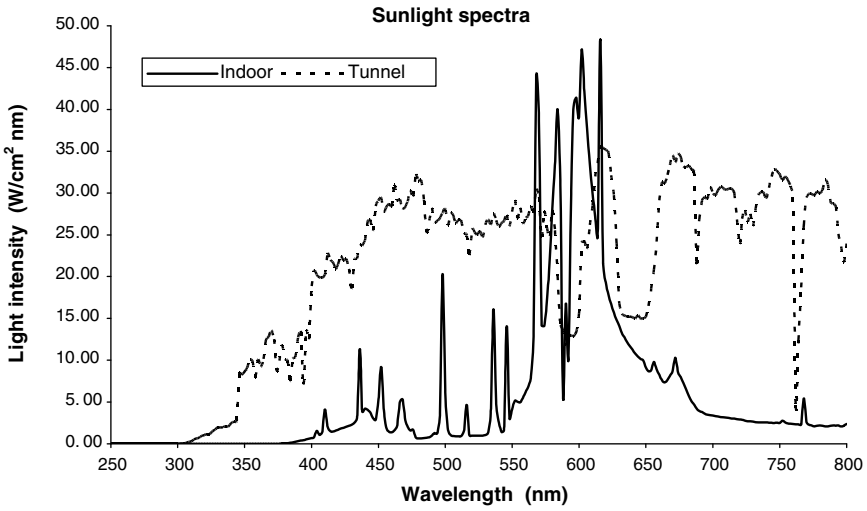

Fig. 1. Irradiance spectra. Solid line, emission spectrum in growth room. Dotted line, irradiance spectrum in growth tunnel, comprising solar radiation filtered through a single layer of fine mesh.

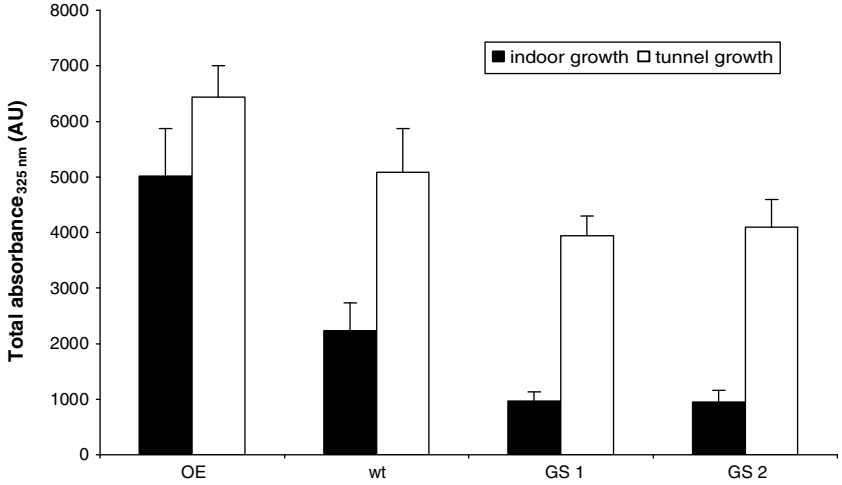

Fig. 2. Estimation of the soluble phenolic content of tomato leaves. HQT-overexpressing plants (OE line), gene-silenced plants (GS-1 and GS-2 line) and wild type plants were raised under indoor (■) or tunnel (□) conditions. Phenolic compounds were extracted using methanol and their total levels were quantified at 325 nm (Nara et al., 2006) using a spectrophotometer. Values were normalised on biomass. Bars indicate standard error (based on 6–10 different leaves originating from different plants). Growth-room-raised antisense plants accumulate significantly less total phenolics than wild type ($P < 0.05$) and HQT-overexpressing plants ($P < 0.01$), and wild type plants accumulate less phenolics than HQT-overexpressing plants ($P < 0.05$). Tunnel-raised antisense plant (GS-1) accumulated significantly less phenolics than HQT-overexpressing plants ($P < 0.05$).

between wild type, gene-silenced and overexpressing lines under these indoor growth conditions was due, predominantly, to different CGA-levels as might have been predicted by modifying HQT expression.

Plants raised under the higher light conditions in the tunnel contained substantially higher levels of soluble, UV-absorbing compounds than plants raised in the growth room (Fig. 2). CGA was the most abundant phenolic compound (56% of total, soluble UV absorbance) that was present in methanolic extracts of wild type plants raised under tunnel conditions. In the gene-silenced lines, CGA-levels were low, and comprised just 22–28% of the soluble phenolics. Tunnel-grown, HQT-overexpressing plants contained similar levels of CGA as wild type plants (Table 1). Rutin, rutin–pentose, flavonoid and tomatine levels increased in the tunnel-grown plants, compared to indoor-grown plants. The increases in the levels of rutin, rutin–pentose, quercetin, kaempferol and tomatine were stronger in the gene-silenced and HQT-overexpressing lines than in the wild type line.

UV-absorbing phenolics play an important role in protecting tissues and cells from harmful UV photons. The maximal efficiency of PSII (F_v/F_m) is commonly used as a non-invasive indicator of damage to the photosynthetic machinery, and conversely, as an indicator of the efficiency of protective systems (Jansen et al., 1998). We found that the maximal efficiency of PSII was very similar for all three lines, irrespective of growth conditions, prior to UV exposure (Fig. 5A and B). This indicated that none of the lines was stressed as a result of altered HQT expression, *per se*. This observation was also consistent with the similar growth performance of these lines.

Exposure of leaf discs, from growth-room-raised plants, to a low UV dose-rate (800 μW cm^{-2}) resulted in a substantial decrease in PSII efficiency. The damaging effects of UV radiation on F_v/F_m were significantly greater ($P < 0.01$) for the HQT gene-silenced plants than for either wild type or HQT-overexpressing plants (Fig. 5A). Under the low UV dose-rate, the WT and HQT-overexpressing lines were similar in terms of their UV-tolerance. It is possible that WT

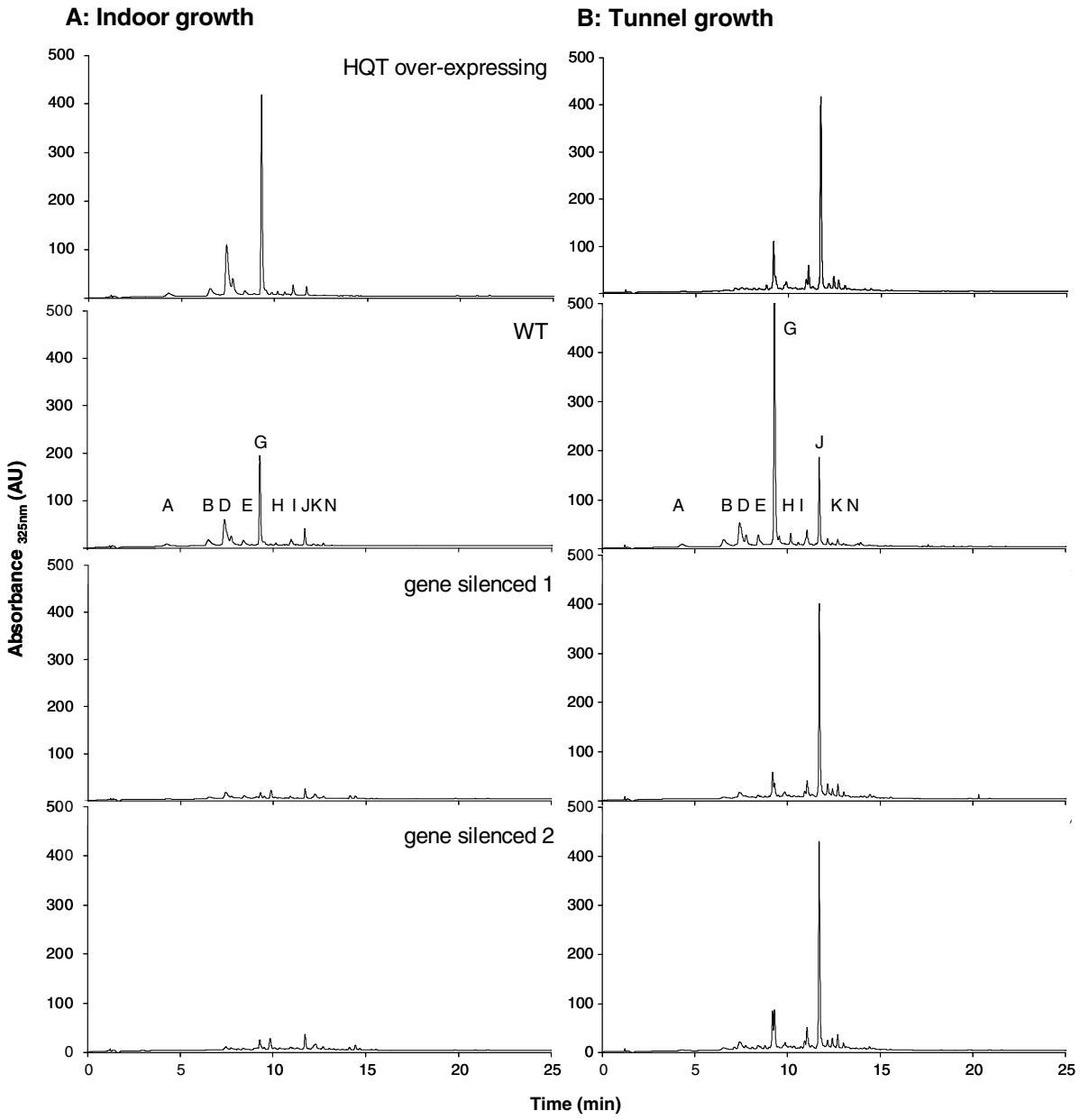

Fig. 3. Visualisation of soluble *S. lycopersicum* phenolics using liquid chromatography. Leaf tissue was homogenized, phenolic compounds were extracted using methanol, and separated using LC. The figure shows representative spectra of growth room (A) and tunnel (B) raised HQT-overexpressing, or gene-silenced plants, as well as wild type. Peak identification; B, chlorogenic acid, retention time 6.55 min; D, chlorogenic acid, retention time 7.42 min; E, chlorogenic acid, retention time 8.41 min; G, chlorogenic acid, retention time 9.30 min; I, Rutin–pentose, retention time 11.07 min; J, Rutin, retention time 11.73 min; K, Quercetin–glucose, retention time 12.17 min; L, Kaempferol–rhamnose–glucose, retention time 12.43 min; M, Dehydro-tomatine, retention time 12.70 min; N, Tomatine, retention time 13.04 min.

and HQT-overexpressing lines differ in terms of phenolic metabolite mediated UV-protection, but that this difference was masked by active repair metabolism (Jansen et al., 1998). Therefore, we also exposed plants to a high, acute dose-rate of UV radiation (4 h 1000 μW cm^{-2}), conditions under which repair metabolism is less likely to diminish accumulated damage. Under these more acute stress conditions, the HQT-overexpressing line (growth-room-raised) was less affected by UV than the WT line (Fig. 6A, $P < 0.01$). The gene-silenced lines were very severely damaged following UV exposure (Fig. 6A).

The increased UV-sensitivity of the growth-room-raised, gene-silenced lines was entirely consistent with the decreased levels of UV-absorbing phenolics in these plants (Fig. 2). The UV-absorbing phenolic pool in tomato leaves is comprised largely of CGA (Table 1). Therefore, under these growth conditions CGA acted to protect plants from UV-damage. The small increase in UV-tolerance in the growth-room-raised HQT-overexpressing line exposed to high, acute dose-rate UV-B is also consistent with increases in CGA (Table 1) and total soluble phenolics (Fig. 2).

To study the relation between CGA-levels and UV-protection in more detail, we repeated the UV-susceptibility assay on plants raised under tunnel conditions. Plants raised under tunnel-growth conditions were notably more UV-tolerant than those raised under growth chamber conditions (compare Fig. 6A and B). This observation is consistent with our measured increases in the overall levels of phenolic compounds in tunnel-raised plants compared to those raised in growth chambers (Fig. 2 and Table 1). Consequently, tunnel-raised plants were challenged with higher UV dose-rates to achieve significant effects on maximal PSII efficiencies. Under these radiation conditions, the HQT-overexpressing plants were

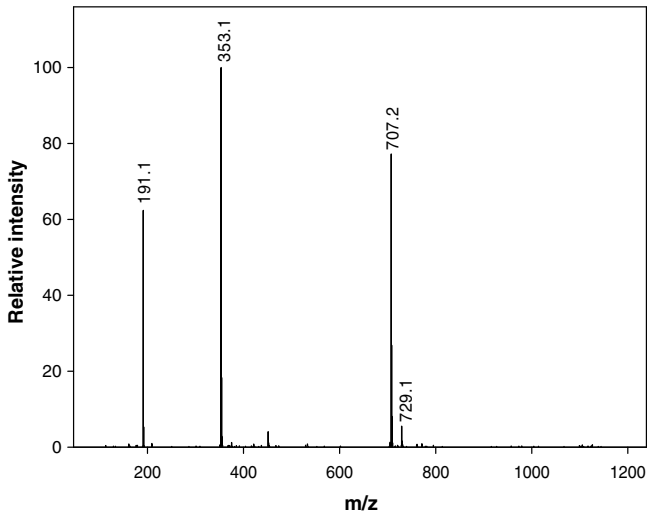

Fig. 4. Identification of main CGA peak in tomato-extracts using LC–MS. The CGA-molecule was characterised by its key mass peaks; m/z = 353 amu is the negative ion formed from chlorogenic acid by loss of a proton, m/z = 707 amu a probable in-source dimer, m/z = 729 amu the sodium adduct of in-source-dimer, m/z = 191 amu fragment formed by loss of caffeate.

as susceptible as the gene-silenced lines and all were more UV-susceptible than wild type control plants grown in the tunnel ($P < 0.025$) (Fig. 5B). Thus, when plants were raised under tunnel conditions, the levels of CGA in the plants appeared to be less important in conferring UV-tolerance, than in growth room-grown plants.

3. Discussion

Overall, CGA-levels were found to be low in HQT-gene-silenced lines, intermediate in wild type and high in HQT-overexpressing plants, indicating a positive correlation CGA accumulation and HQT expression (Fig. 3, Table 1). The consequences of altered HQT expression on CGA accumulation were particularly clear in growth chamber-raised plants. Previously, we have shown that gene-silenced plants still contained a small amount of HQT mRNA, which was detected on Northern blots (Niggeweg et al., 2004). Consistently, we found that the gene-silenced plants accumulated a small amount of CGA. The overexpression plants accumulated 2.5-fold more CGA than wild type controls (Table 1). Thus, we conclude that HQT exercises significant control over the flux towards CGA in tomato leaves under growth room conditions. These data are in agreement with those of Niggeweg et al. (2004), who showed a link between HQT expression levels and CGA accumulation using glasshouse-raised plants.

We have shown that growth conditions exert an important influence on the levels of CGA that accumulate in leaves. Overall, we found that CGA accumulation in tomato leaves increased upon transfer of plants from growth room to tunnel. However, this was not the case for the HQT-overexpressing plants which accumulated very similar levels of CGA as the wild type under ambient light conditions in the tunnel (Table 1). Thus, HQT activity is not rate limiting for CGA accumulation under tunnel-growth conditions, where high levels of phenolic compounds accumulate (Fig. 2). This result is somewhat surprising as we had anticipated that, under low light conditions, PAL-activity, and hence levels of phenylpropanoid precursors, would be limiting CGA accumulation in growth-room-raised plants (Bate et al., 1994; Howles et al., 1996). Yet, the observed effectiveness of HQT-manipulation under low light (Table 1), suggests that the activity of this step contributes significantly to determining the flux to CGA under growth room conditions. HQT may compete effectively with other branches of the phenylpropanoid pathway for the supply of precursors, particularly under low light conditions. Yet, under indoor growth conditions we could not discern any clear relationship between CGA accumulation and the accumulation of rutin, kaempferol, quercetin or tomatine (Table 1), which were present in all lines at low levels. However, levels of these metabolites increased when plants were transferred to the natural light conditions in the tunnel. This was especially the case for the gene-silenced and HQT-overexpressing plants (Table 1). For example, gene-silenced plants raised under tunnel conditions contained relatively low levels of CGA but high levels of the glycosylated-quercetin, rutin (on average twice as much as in the wild type; Table 1). Similarly, levels of kaempferol, quercetin and tomatine were, on average, twice as high as in the wild type. This might reflect a compensatory and/or feedback mechanism, whereby the suppression of CGA synthesis and the accumulation of one or several CGA precursors from general phenylpropanoid metabolism lead to increased accumulation of rutin in the tunnel-raised gene-silenced plants. However, tomatine is a glycoalkaloid, the synthesis of which does not involve the phenylpropanoid pathway. Furthermore, compensatory mechanisms do not explain why the levels of rutin, kaempferol, quercetin and tomatine were also relatively high in HQT-overexpressing plants that contained similar levels of CGA as wild type plants.

Plants raised under natural sunlight conditions contained higher overall levels of soluble phenolics than growth chamber-raised plants. These plants are also relatively UV-tolerant (compare Fig. 6A and B). This confirmed the often reported, general link between UV-tolerance and phenolic content (Jansen et al., 1998).

Table 1
Content of the main phenolic compounds present in methanolic extracts of tomato plants with differing HQT expression patterns

		Relative peak surface area (standard error of the mean)									
		B	D	E	G	I	J	K	L	M	N
Indoor growth	WT	*188 (37)*	*541 (90)*	*149 (28)*	*805 (234)*	115 (31)	169 (30)	34 (5)	17 (2)	34 (7)	11 (2)
	GS 1	*67 (4)*	*152 (22)*	*81 (15)*	*164 (87)*	50 (15)	215 (64)	60 (18)	18 (3)	48 (5)	26 (8)
	GS 2	*84 (21)*	*238 (92)*	*89 (16)*	*152 (74)*	29 (4)	161 (23)	26 (3)	17 (3)	47 (7)	20 (3)
	OE	*270 (29)*	*925 (152)*	*182 (6)*	*2914 (621)*	228 (47)	134 (7)	36 (3)	10 (3)	28 (3)	11 (1)
Tunnel-growth	WT	*264 (34)*	*840 (208)*	*208 (27)*	*1786 (523)*	234 (36)	824 (179)	86 (19)	48 (8)	79 (14)	30 (7)
	GS 1	*88 (17)*	*196 (23)*	*87 (11)*	*414 (90)*	241 (18)	2003 (286)	168 (33)	149 (13)	140 (20)	65 (9)
	GS 2	*110 (13)*	*258 (66)*	*78 (19)*	*637 (196)*	246 (31)	2011 (281)	142 (21)	159 (27)	139 (15)	60 (11)
	OE	*148 (37)*	*623 (301)*	*181 (101)*	*1999 (638)*	358 (25)	2305 (544)	167 (34)	142 (28)	143 (29)	68 (15)

Integrated peak surface areas are indicated for wild type (WT), HQT-gene-silenced (GS-1 and GS-2) and HQT-overexpressing lines (OE). Given between brackets is the standard error of the mean for each peak. Chlorogenic acid isomers are in italics.
B, chlorogenic acid, retention time 6.55 min; D, chlorogenic acid, retention time 7.42 min; E, chlorogenic acid, retention time 8.41 min; G, cacid, retention time 9.30 min; I, Rutin–pentose, retention time 11.07 min; J, Rutin, retention time 11.73 min; K, Quercetin–glucose, retention time 12.17 min; L, kaempferol–rhamnose–glucose, retention time 12.43 min; M, dehydro-tomatine, retention time 12.70 min; N, Tomatine, retention time 13.04 min.

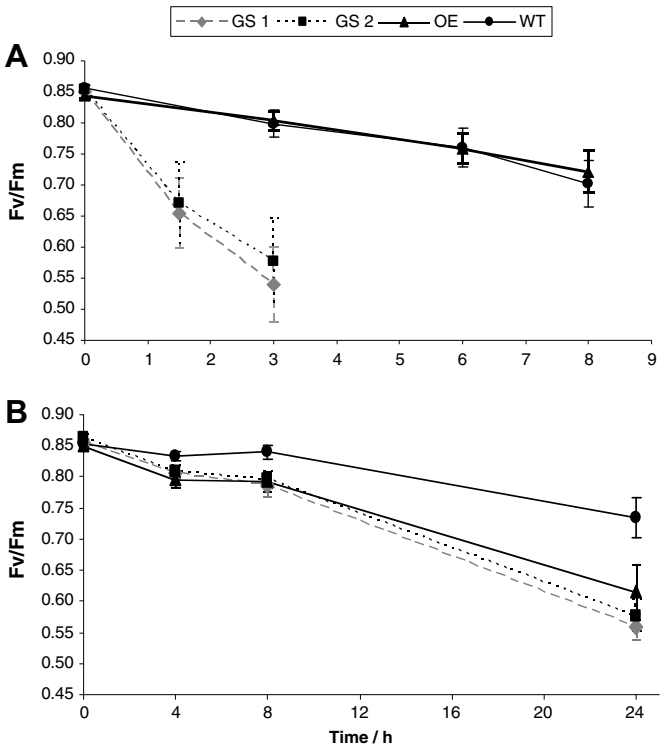

Fig. 5. Chlorophyll fluorescence measurements of UV-B exposed leaves. Leaves of growth-room-raised plants were exposed for up to 8 h to a UV-B dose-rate of 800 μW/cm^2 (5A). Leaves of tunnel-raised plants were exposed for up to 24 h to a UV-B dose-rate of 1000 μW/cm^2 (B). HQT-overexpressing line OE (▲), HQT-gene-silenced lines GS-1 (♦) and GS-2 (■) and wild type (●). The relative variable fluorescence (F_v/F_m) was measured with a PAM chlorophyll fluorometer following 10 min of dark adaptation. $N = 20$; Standard errors of the mean are shown. For growth room plants; wild type and overexpressing lines were significantly less impacted by UV-B than gene-silenced lines ($P < 0.01$) (A). For tunnel plants; wild type was significantly less impacted by UV-B than gene-silenced lines (line GS-1, $P < 0.01$; line GS-2, $P < 0.025$) (B).

Our data also indicate the effectiveness of CGA in UV-protection, particularly for plants raised under low light conditions in a growth room. However, a more detailed analysis of the link between UV-absorbing soluble compounds and UV-protection indicated a more complex relationship between phenylpropanoid metabolites and UV-tolerance. Particularly, we noted that under tunnel conditions the HQT-overexpressing plants were not more UV-tolerant than similarly raised wild type plants, (Fig. 5B), despite the former containing significantly higher levels of flavonoids (rutin and rutin-derivatives; Table 1). Optical saturation of the protective screening response is unlikely, as the increase in the level of UV-absorbing pigments upon transfer of OE plants to the tunnel (Fig. 2) was associated with an increase in UV-tolerance in that line (Fig. 6A and B). Possibly, the specific composition of the phenolic profile is also important in determining overall UV-tolerance. We have noted changes in the rutin:CGA ratio, with this ratio being particularly high in gene-silenced and HQT-overexpressing tunnel-raised plants. At the moment we do not understand the physiological relevance of this particular change in phenolic profile. However, we recognise at least three possible explanations of how shifts in the phenolic profile lead to changes in UV-sensitivity.

(1) Phenolics differ with respect to their ROS-scavenging capabilities. ROS are key components of UV radiation stress (Jansen et al., 1998) and scavenging of reactive molecules prevents UV-damage. Hydroxylated phenolics in particular, are effective ROS scavengers (Wilson et al., 1998). However, we note that CGA and rutin have very similar antioxidant capacities (Kim et al., 2002). Therefore, we consider it unlikely that differences in overall ROS-scavenging capability are responsible for the observed difference in UV-protection in the tomato lines we studied.

(2) Different phenolics accumulate at different (sub-cellular) locations. It has recently been hypothesised that protective UV screening is based on a three tier system that comprises accumulation of UV screening pigments at the level of the leaf, the cell and the organelle (Meijkamp, 2006). The efficiency of the UV screening will depend on, amongst other things, the balance between these three tiers, and therefore ultimately the phenolic profile. All the phenolics we measured were soluble, and some differences in UV-tolerance may be attributable to other differences between the lines and growth conditions, particularly differences in cell wall-associated phenolics.

(3) Phenolic profiles comprise soluble, ionically bound and covalently bound compounds. One of the HQT substrates, caffeoyl CoA, can also serve as a precursor in the formation of UV-absorbing lignin and lignin-like polymers in the cell wall (Niggeweg et al., 2004). Such polymers have been found to be upregulated in UV-exposed plants (Rozema et al., 1997) and are associated with UV-protection (Jansen et al., 1998). Thus, a consequence of the genetic upregulation of HQT activity might be a decrease in the availability of its substrate, caffeoyl CoA, and a consequent decrease in polymerized, lignin-like, UV screening phenolics in the cell wall.

Our study emphasises the plasticity of the phenylpropanoid pathway with its many branches. In particular, our data reveal the re-direction of the flow of phenolic precursors from CGA to rutin in both HQT-overexpressing and gene-silenced tomato lines raised under tunnel conditions. This shift in phenolic profile must reflect physiological regulatory mechanisms that control the balance between the different branches of the phenylpropanoid pathway in response to environment. The shift in phenolic profile has consequences for UV-protection. This particular point is rarely acknowledged. There is overwhelming evidence for a link between overall levels of soluble, UV-absorbing pigments and UV-protection (Hollosy, 2002; Jansen et al., 1998; Jordan, 1996). However, our data (Figs. 2, 5 and 6), show a divergence between UV-protection and overall phenolic contents in the case of the HQT-overexpressing plants. We consider it unlikely that the genetic modulation of HQT expression triggers changes in completely unrelated UV-repair or defence mechanisms, therefore, we conclude that the differences in UV-protective capacity in plants raised under the same growth conditions, are determined by both overall phenolic accumulation and the specific phenolic profile.

4. Experimental

4.1. Plant material

The generation of *S. lycopersicum* cv. Moneymaker transgenics, with altered expression of the gene encoding HQT, has been detailed previously (Niggeweg et al., 2004). The full-length cDNA encoding tomato HQT was subcloned in frame in the *Nco*I and *Sma*I-sites of the expression vector pJIT166 under the control of the 35S-promotor. The expression cassette containing the promoter, the cDNA and the CaMV terminator was then cloned in the binary vector pBIN19. The resulting plasmid pTOM was used for transformation of tomato plants to yield HQT-overexpressing lines (OE lines). In a few cases transformation with the endogenous gene led to gene-silencing (GS lines). In a previous paper, the OE and GS-2 lines were referred to as tom9 and tom5, respectively

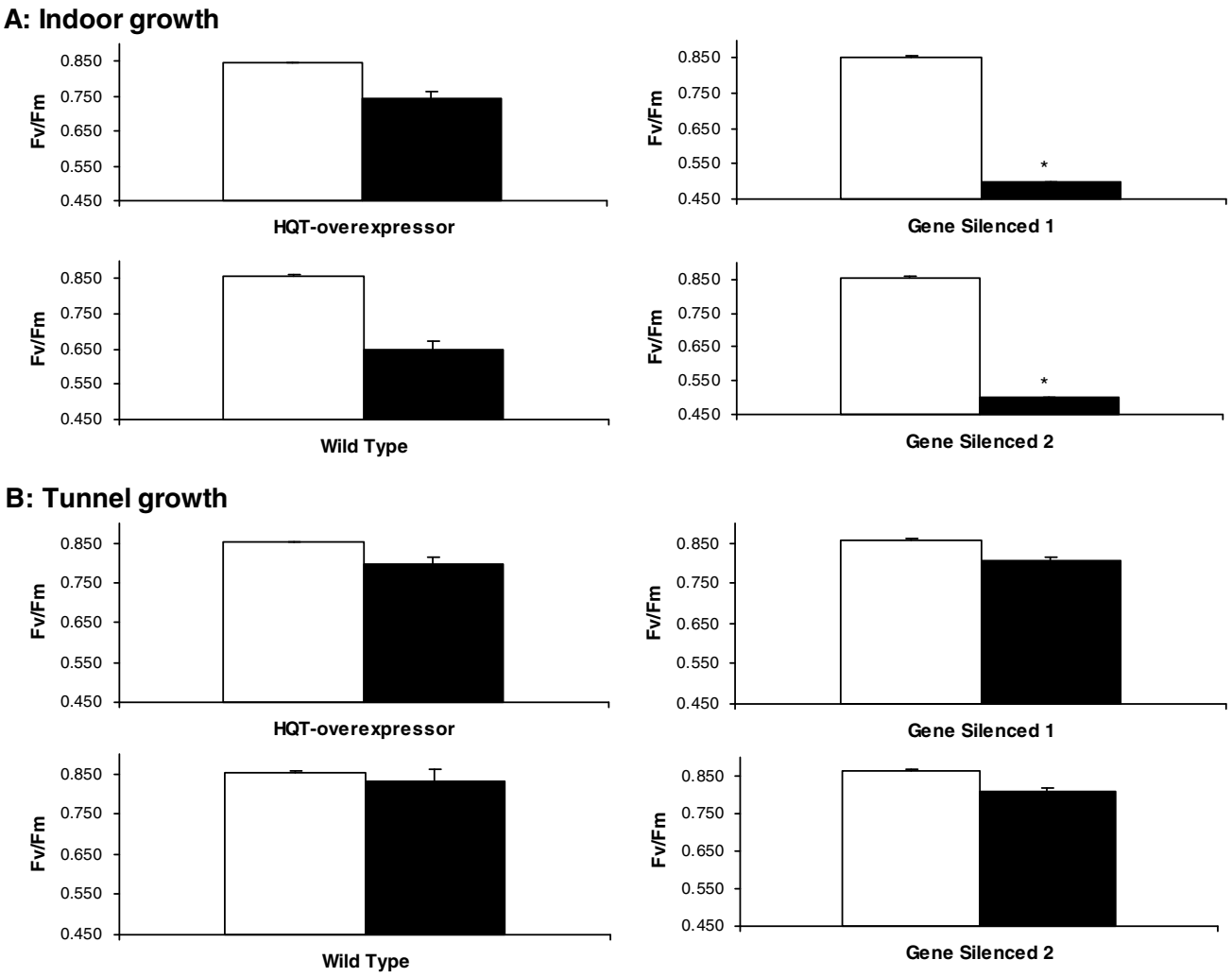

Fig. 6. Chlorophyll fluorescence measurements of UV-B exposed leaves. Leaves of the HQT-overexpressing line OE, HQT-gene-silenced lines GS-1 and GS-2 and the wild type were exposed for 4 h to a UV-B dose-rate of 1000 μW/cm^2. Plants were raised under either indoor (A) or tunnel conditions (B). The relative variable fluorescence (F_v/F_m) was measured before (□) and after (■) UV-B exposure with a PAM chlorophyll fluorometer following 10 min of dark adaptation. * Indicates that F_v/F_m levels dipped below biologically relevant values within period of exposure. $N = 20$; Standard errors of the mean are shown.

(Niggeweg et al., 2004). GS-1 is an independent primary transformant that was found to be very similar to GS-2. Both the gene-silenced and the HQT-overexpressing tomato lines were analysed by Northern blotting, and this confirmed the differential expression patterns (Niggeweg et al., 2004).

The tomato lines used in these studies were rooted-cuttings from the original transformants that have been described earlier (Niggeweg et al., 2004). Cuttings were grown on multipurpose compost (Westland Horticulture, Winsford, UK), in growth rooms at 21 °C, under 16 h light/8 h dark cycles, and at a relative humidity of 70%, or in tunnels with maximal temperatures of 25 °C, a day-length of circa 16 h, and ambient light and humidity conditions. Light intensities in the growth room were 250 μmol m^{-2} s^{-1}. Irradiance conditions during growth were measured using an optronic OL754 spectroradiometer.

4.2. Phenolic extraction and analysis

The accumulation of phenolic compounds was assessed by measuring the UV absorbance of methanolic extracts at 325 nm. Leaf discs were ground to a fine powder and 70% methanol (MeOH, HPLC grade) was added in the ratio of 300 μL MeOH to 30 mg tissue. Subsequently, a further 100 μL H_2O (per 30 mg tissue) was added and the samples were centrifuged for 30 s at 14,000 rpm. The supernatant was stored in the dark at a temperature of −80 °C.

Phenolic compounds were analysed by liquid chromatography–mass spectrometry (LC–MS) using an Agilent 1100 LC system equipped with diode array UV and single quadrupole 1100MSD detectors. Quantification was by area of UV peaks, and identification was by mass. To confirm identification, selected samples were also analysed using a Surveyor LC system (Thermo Fisher) equipped with a DecaXPplus ion trap mass spectrometer. In both cases, separation was on a Luna 3 μ C18(2) column, 100 × 2 mm (Phenomenex, Macclesfield, UK) using a gradient of acetonitrile (B) versus 0.1% formic acid in water (A), and the following gradient, run at 250 μL min^{-1}: 0 min 95% A, 5% B; 15 min 50% A, 50% B; 17 min: 30% A, 70% B; 23 min: 30% A, 70% B, 25 min: 95% A, 5% B; 33 min: 95% A, 5% B. Ionisation was by negative electrospray, and in the ion trap, MS^2 and MS^3 data were collected at 35% collision energy and an isolation width of 4 amu.

4.3. UV-B exposure conditions

UV-treatments consisted of exposure of tomato leaf discs to UV radiation. Leaf discs were taken from young, but fully expanded leaves, and were floated on water (adaxial site up) in a Petri dish

covered with cellulose acetate filter. UV-B was generated using Philips TL12 tubes (Philips, Eindhoven, The Netherlands). Under our experimental conditions, tunnel-raised plants were less UV-sensitive than growth room-raised plants. Therefore, exposure times and irradiance conditions were adjusted accordingly. Leaf discs were exposed to a low (8 h of 8.0 W m^{-2}) or a high UV dose-rate (8 h of 10 W m^{-2}). These dose-rates have no direct environmental relevance, but rather were used to determine the relative level of UV-tolerance of the different lines. The dose-rate comprises radiation in the spectral range between 295 nm and 345 nm. UV-C wavelengths were blocked using cellulose acetate filters (95 μm, Kunststoff-Folien-Vertrieb GmbH, Hamburg, Germany). No additional background of photosynthetically active radiation (PAR) was applied during the UV-treatment. UV-fluences were measured using a PMA2000 (Solar light, Philadelphia, USA).

UV-mediated damage to the photosynthetic machinery was assessed by measuring the photosynthetic efficiency of PSII using a modulated fluorometer (PAM, Walz, Effeltrich, Germany). The minimal fluorescence (F_0), maximal fluorescence (F_m) and the variable fluorescence ($F_v = F_m - F_0$) were measured using the saturated pulse method (van Kooten and Snel, 1990). The photochemical yield of open PSII reaction centres, commonly known as the relative variable fluorescence, was calculated as F_v/F_m, reflecting the maximal photosynthetic efficiency of PSII measured in dark adapted tissue (10 min).

Statistics were carried out in SAS. The Shapiro–Wilk test was used to test for normal distributions. To identify significant differences between two samples, the Student *t*-test was performed and differences in F_v/F_m values were tested using Anova.

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