Expression and Functional Analyses of the Plastid Lipid-Associated Protein CHRC Suggest Its Role in Chromoplastogenesis and Stress

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Chromoplastogenesis during flower development and fruit ripening involves the dramatic overaccumulation of carotenoids sequestered into structures containing lipids and proteins called plastid lipid-associated proteins (PAPs). CHRC, a cucumber (*Cucumis sativus*) PAP, has been suggested to be transcriptionally activated in carotenoid-accumulating flowers by gibberellin (GA). *Mybys*, a MYB-like trans-activator identified here, may represent a chromoplastogenesis-related factor: Its expression is flower specific and parallels that of *ChrC* during flower development; moreover, as revealed by stable ectopic and transient-expression assays, it specifically trans-activates *ChrC* promoter in flowers accumulating carotenoids and flavonoids. A detailed dissection of *ChrC* promoter revealed a GA-responsive element, gacCTCcaa, the mutation of which abolished *ChrC* activation by GA. This cis-element is different from the GARE motif and is involved in *ChrC* activation probably via negative regulation, similar to other GA-responsive systems. The GA responsiveness and MYBYS floral activation of the *ChrC* promoter do not overlap with respect to cis-elements. To study the functionality of CHRC, which is activated in vegetative tissues similar to other PAPs by various biotic and abiotic stresses, we employed a tomato (*Lycopersicon esculentum*) plant system and generated RNAi-transgenic lines with suppressed LeCHRC. Transgenic flowers accumulated approximately 30% less carotenoids per unit protein than controls, indicating an interrelationship between PAPs and flower-specific carotenoid accumulation in chromoplasts. Moreover, the transgenic LeCHRC-suppressed plants were significantly more susceptible to Botrytis cinerea infection, suggesting CHRC’s involvement in plant protection under stress conditions and supporting the general, evolutionarily preserved role of PAPs.

Carotenoid-accumulating plastids, the chromoplasts, are responsible for the yellow, orange, and red colors of flower parts, fruits, old leaves, and some roots in various plant species. The pigment accumulates in structures composed of lipids and proteins, allowing sequestration/stabilization of the large amounts of carotenoids. Although any plastid can be converted to chromoplasts, they are usually derived from chloroplasts during fruit maturation and flower development. Studies on the molecular organization of carotenoid-overaccumulating structures have led to the identification and characterization of several nuclear genes encoding carotenoid-associated proteins (Deruere et al., 1994; Bartley and Scolnik, 1995; Vishnevetsky et al., 1996). In fruit and flower tissues, spatial and temporal expression of these genes is regulated essentially at the transcriptional level. In leaves, their expression under standard greenhouse conditions is very low (Deruere et al., 1994; Vishnevetsky et al., 1999a; Murphy, 2004). The first gene involved with carotenoid storage, termed fibrillin (*Fib*), was identified in red fruit chromoplasts of *Capsicum annuum* (Newman et al., 1989; Deruere et al., 1994). In floral tissues, a 35-kD carotenoid-associated protein (CHRC) was characterized in chromoplasts of yellow cucumber (*Cucumis sativus*) corollas (Vainstein et al., 1994; Vishnevetsky et al., 1996). At both the protein and transcript levels, *Fib* and *ChrC* accumulation was shown to parallel carotenoid accumulation and fibril development in fruits and corollas (Deruere et al., 1994; Vishnevetsky et al., 1996). In fruit and flower tissues, spatial and temporal expression of these genes is regulated essentially at the transcriptional level. In leaves, their expression under standard greenhouse conditions is very low (Deruere et al., 1994; Chen et al., 1998; Vishnevetsky et al., 1999b). In floral tissue, GA3 plays a critical role in chromoplastogenesis: It leads to enhanced carotenoid accumulation as well as to transcriptional activation of *ChrC* expression. The response to GA was localized to a 290-bp fragment within the *ChrC* promoter that does not contain known GA-responsive elements (GARE/CARE; Gubler et al., 1999; Vishnevetsky et al., 1999b; Sutoh and Yamauchi, 2003).

Modulation of the composition and amount of carotenoids accumulating in chromoplasts affects the expression of *ChrC*. Nevertheless, its flower-specific transcriptional activation is not strictly dependent on
accumulated carotenoids, but rather on an unidentified regulatory factor related to chromoplastogenesis. This proposal was based on the observation that in chromoplast-lacking flavonoid-accumulating flowers, ChrC promoter is not active, as revealed by transient-expression experiments (Vishnevetsky et al., 1999b).

Numerous CHRC/Fib homologs have been identified in different plant systems, as well as in plastids other than chloroplasts, and have therefore been collectively termed plastid lipid-associated proteins (PAPs; Pruvot et al., 1996; Kessler et al., 1999; Kim et al., 2001). Recently, using bioinformatics/genomic tools, several Arabidopsis (Arabidopsis thaliana) homologs have been identified and phylogenetically characterized (Laizet et al., 2004). The expression of PAPs in nonchromoplastogenetic tissues led to the suggestion that they are involved not only in the storage of carotenoids but also in the general sequestration of hydrophobic compounds such as lipids. Supporting this hypothesis, Ting et al. (1998) and Hernandez-Pinzon et al. (1999) showed that CHRC homologs are components of tapetal lipid bodies in Brassica napus. Within the chloroplast of pea (Pisum sativum), the CHRC homolog PG1 was identified on the surface of lipid-containing structures—plastoglobules, which are the origin of carotenoid-accumulating structures in chloroplasts (Kessler et al., 1999).

The accumulation of PAPs in plastids as well as the biogenesis of structures that sequester hydrophobic compounds are accelerated by various stresses (Murphy, 2004). For example, the expression of Fib is transcriptionally up-regulated by both biotic and abiotic stresses (Chen et al., 1998; Kuntz et al., 1998; Manac’h and Kuntz, 1999; Langenkamper et al., 2001). Furthermore, gene expression and protein accumulation of the potato (Solanum tuberosum) PAP homolog CDSP34 in leaf chloroplasts are induced by different osmotic and oxidative stress conditions (Pruvot et al., 1996; Gillet et al., 1998; Langenkamper et al., 2001). This PAP has been implicated in the modulation of photosynthetic efficiency and in the dissipation of excess absorbed light energy (Monte et al., 1999). Similarly, the expression of B. napus and Arabidopsis PAPs is regulated by various abiotic stresses (Kim et al., 2001; Laizet et al., 2004). Moreover, Rey et al. (2000) have shown that overexpressing Fib in tobacco (Nicotiana tabacum) improves plant performance under stress conditions. Hence, it is apparent that in addition to chromoplastogenesis, PAPs play an important general role in the sequestration of hydrophobic molecules, a process that may be essential for plant survival under stress.

Here, we identified a MYB-like factor, MYBYS, which specifically trans-activates ChrC promoter in floral tissue irrespective of chromoplastogenesis, and dissected the ChrC promoter to identify a GA-responsive element, gacCTCcaa, involved in ChrC activation, probably via negative regulation. To characterize CHRC’s role in vegetative tissues as well, we generated transgenic tomato (Lycopersicon esculentum) plants with RNAi-suppressed LeCHR C. Using these plants, we showed the involvement of CHRC not only in flower-specific carotenoid accumulation in chloroplasts but also in susceptibility to Botrytis cinerea infection.

RESULTS

MYB-Like Factor MYBYS Activates the ChrC Promoter

We previously found that in floral tissues, expression of ChrC depends on a factor related to chromoplastogenesis and that it is transcriptionally up-regulated by the hormone GA3 (Vishnevetsky et al., 1999b). With the aim of identifying this factor, we screened a cDNA expression library from cucumber flowers with a 137-bp segment of the ChrC promoter that is responsive to chromoplastogenesis and floral signals. A MYB-like transcription factor, having a typical DNA-binding domain containing the helix-turn-helix conserved R2 and R3 repeats, was identified and termed MYBYS (MYB-like, GenBank DQ311672). C-terminal to the DNA-binding domain, MYBYS contains a motif of amino acids (Gln and Pro) that is frequently associated with activation domains (Fig. 1A). Analyses of mybys expression at the RNA level in floral tissue revealed that it accumulates in parallel to flower development, up to anthesis, at which stage no transcript was revealed. No expression was detected in leaf tissues. This spatial and temporal pattern of expression is essentially identical to that of ChrC (Fig. 1B). To assess whether the identified MYBYS can activate ChrC promoter in floral tissue irrespective of chromoplastogenesis, we co-bombarded mybys, under the regulation of cauliflower mosaic virus (CaMV) 35S (35S:MYBYS), with 137ChrC:β-glucuronidase (GUS) or ChrC:GUS (137 bp or 3,500 bp of the ChrC promoter fused to GUS, respectively) into petunia (Petunia hybrida) corollas. Trans-activation of ChrC promoter by MYBYS was revealed through the expression of GUS in chromoplast-lacking petunia corollas following co-bombardment. No GUS expression was observed in petunia corollas following bombardment with 137ChrC:GUS or ChrC:GUS alone, or when they were co-bombarded with an unrelated MYB driven by CaMV 35S (35S:PAP or 35S:LeC; Ben-Meir, 2003; Fig. 2A). Nor was GUS expression observed when 35S:MYBYS was co-bombarded with a minimal TATA-box promoter fused to GUS or with the unrelated promoter construct glutathione S-transferase: GUS (Zenvirt, 2000). The same results, i.e. trans-activation of 137ChrC:GUS or ChrC:GUS specifically by MYBYS, were obtained when other flowers that do not accumulate chloroplasts, carnation (Dianthus caryophyllus) and gypsophila (Gypsophila paniculata), were used in the transient-expression assays (data not shown). In leaves, MYBYS was not sufficient for ChrC:GUS trans-activation.

To further evaluate the activity of MYBYS in planta, we generated transgenic tomato plants expressing it under the CaMV 35S promoter. Tomato plants were used as they are highly amenable to transformation...
Expression of mybys in young corollas of transgenic (T2-generation) plants, as compared to control nontransgenic ones, was confirmed by RNA-blot analysis (Fig. 2B, bottom). Bombardment of young green flower corollas with 137ChrC:GUS or ChrC:GUS yielded GUS expression in mybys-transgenic flowers but not in control plants (Fig. 2B). It should be noted that at this early stage of flower development, endogenous PAP is not yet expressed (Vishnevetsky, 1999).

GA-Responsive Element within the ChrC Promoter

GA₃ has been shown to promote chromoplastogenesis and CHRC accumulation in floral tissue (Vainstein et al., 1994). The accumulation of CHRC was shown to be regulated at the RNA level (Vishnevetsky et al., 1997). To detail GA₃-responsive cis-elements in the ChrC promoter, we constructed a series of vectors (Fig. 3A) aimed at characterizing the 212-bp region shown to be responsible for the response to GA₃ (Vishnevetsky et al., 1994).
et al., 1999b). One construct contained the \textit{ChrC} promoter (3,500 bp) without the 212-bp region, fused to a GUS reporter gene (\textit{D}212ChrC:GUS). The others contained 212 (from 290 to 78), 137 (215 to 78), 63 (141 to 78), or 46 (124 to 78) bp of \textit{ChrC} promoter fused via a minimal TATA-box promoter to the GUS reporter gene. All constructs were delivered by particle bombardment to young cucumber flower buds subjected to GA3 or water treatment. Construct containing CaMV 35S promoter fused to green fluorescent protein (GFP) was cobombarded and used to normalize the transient GUS expression results. Whereas the 212-, 137-, and 63-bp fragments of \textit{ChrC} promoter were responsive to GA3, showing 3- to 4-fold higher GUS activity in GA3 versus water treatment, the 46-bp fragment was not affected by GA3 (Fig. 3A). Interestingly, this 46-bp fragment drove high GUS expression in control, nontransgenic flowers (WT) were also bombardmed with \textit{ChrC}:GUS and histochemically analyzed (top). Accumulation of mybys in 35S:MYBYS transgenic tomato flowers (independent transgenic lines 4, 10, and 110) is shown in the bottom section. RNA-blot analysis was performed using radiolabeled 3' mybys as a probe. A nontransgenic line (WT) and a transgenic line with no expression of mybys (line 3) were used as controls.

**Figure 2.** MYBYS transcription factor specifically activates the \textit{ChrC} promoter. A. Histochemical visualization of GUS activity in petunia flowers bombarded with \textit{ChrC}:GUS alone, or \textit{ChrC}:GUS or 137\textit{ChrC}:GUS (containing GUS driven by 3,500 or 137 bp of the \textit{ChrC} promoter, respectively) cobombarded with 35S:MYBYS. In control cobombardment experiments, MYBYS was replaced by another MYB factor, PAP, which regulates the anthocyanin pathway (35S:PAP). B. Histochemical visualization of GUS activity in young green transgenic tomato flowers constitutively expressing 35S:MYBYS (line 10) following bombardment with \textit{ChrC}:GUS. As a control, nontransgenic flowers (WT) were also bombardmed with \textit{ChrC}:GUS and histochemically analyzed (top). Accumulation of mybys in 35S:MYBYS transgenic tomato flowers (independent transgenic lines 4, 10, and 110) is shown in the bottom section. RNA-blot analysis was performed using radiolabeled 3' mybys as a probe. A nontransgenic line (WT) and a transgenic line with no expression of mybys (line 3) were used as controls.
To further evaluate the GA-responsive cis-elements, we generated four mutations in the 18-bp region by replacing six bases, with three-base gaps between each of the four replacements (Fig. 4A). One construct (MG1) containing a mutation between \(2^{135}\) and \(2^{133}\), upon delivery to young flower corollas, yielded similarly high GUS expression in both water- and GA3-treated corollas, whereas other constructs, including the one with a mutation between \(2^{135}\) to \(2^{130}\) (MG2), were still responsive to GA3 in a manner similar to that of the control nonmutated promoter (Fig. 4B). These results point to the three bases, CTC, between \(2^{138}\) and \(2^{136}\) as necessary elements for the response of ChrC promoter to GA3 activation. It should be noted that none of these analyzed mutations in the 18-bp region affected activation of theChrC promoter by MYBYS, suggesting that this trans-factor acts through different cis-elements present in the promoter.

**Dual Role of CHRC: Carotenoid Accumulation and Stress Responses**

Various stresses, as shown in several plant systems, affect the accumulation of PAPs. For example, while under normal growth conditions PAP expression is specific for carotenoid-overaccumulating organs, biotic and abiotic stress conditions have been shown to activate their expression in additional tissues (Murphy, 2004). Also, analyses of ChrC promoter activity, using a ChrC:GUS transient-expression assay, revealed that it is induced in heat shock-treated cucumber leaves (Fig. 5A). Transcriptional activation of ChrC expression was also revealed following bombardment of powdery mildew-infected leaves. No GUS expression was detected in control, room temperature-treated or uninfected leaves (Fig. 5A). Moreover, endogenous transcript levels of ChrC in stressed leaves, both heat shock-treated and powdery mildew-infected, were strongly up-regulated (Fig. 5B).

To allow functional analyses of CHRC, which is responsive to numerous developmental and environmental cues, we cloned its tomato homolog with the aim of generating transgenic plants with suppressed CHRC expression. Tomato plants provide a useful research system because in addition to bearing chloroplasts accumulating CHRC homolog (Pozueta-Romero et al., 1997), their expressed sequence tag (EST) databases are elaborate and their transformation protocols...
well defined. Furthermore, in the tomato system, Fib promoter and endogenous PAP homolog have been shown to be activated by stresses in a manner similar to that of ChrC in cucumber tissues (Fig. 5; Kuntz et al., 1998; Langenkamper et al., 2001). Based on the partial sequence of the tomato homolog (Pozueta-Romero et al., 1997), we cloned the complete cDNA sequence of \textit{LeChrC} (Fig. 6; GenBank DQ310151). Analyses of \textit{LeChrC} expression at the RNA level revealed spatial and temporal regulation, mimicking that of \textit{ChrC} in cucumber flowers and leaves (data not shown). It should be noted that \textit{LeChrC} BLAST search in The Institute for Genomic Research (TIGR) database revealed one homolog, TC162898, putatively coding for PAP with 37% identity at the amino acid level.

The RNAi approach was employed to generate transgenic tomato plants with suppressed \textit{LeCHRC}. To this end, we used a 5’ partial sequence of \textit{LeChrC}; when analyzed by BLAST against all tomato ESTs in the TIGR database (default parameters), this fragment did not reveal the second TC162898. Following transformation, the regenerated plants surviving selection on kanamycin were screened by PCR and characterized using RNA-blot analysis to evaluate suppression of \textit{LeChrC} (Fig. 7A). The suppression of \textit{LeCHRC} in T2-generation plants was further confirmed using RNA- and western-blot analyses (Fig. 7, B and C). Analysis of pigment level in corollas of independent T2 lines with suppressed \textit{LeCHRC} revealed an approximately 30% reduction in carotenoid level per unit protein as compared to their level in control corollas, nontransgenic lines, or transgenic lines with no suppression of \textit{LeCHRC} (Table I), indicating an effect of PAP on pigment accumulation. It should be noted that carotenoid levels per unit protein in leaves were essentially the same in transgenic and control plants (Table I).
Over the course of several years of plant propagation, we observed that transgenic tomato lines with suppressed LeCHRC are highly susceptible to *B. cinerea* infection as compared to either control nontransgenic tomato or tomato transgenic for different, unrelated genes. To evaluate the susceptibility of LeCHRC-suppressed plants to *B. cinerea* infection, leaves were detached from these and control nontransgenic plants and inoculated with the conidia. Disease severity in leaves of plants with suppressed LeCHRC expression was 2- to 3-fold higher than that in control plants (Table II). To further evaluate this susceptibility, a conidial suspension was applied to leaves and stems of 1-month-old plants. Inoculated plants were then grown in a growth chamber (Table III; Fig. 8A). Three days after inoculation, there was no significant difference in the size of the necrotic lesions on leaves of transgenic LeCHRC-suppressed versus control plants. However, during the following 3 d of growth, lesions on the transgenic leaves increased rapidly, reaching approximately twice the size of controls. A significant difference was also observed in the necrotic lesions developed...
on stems of transgenic LeCHRc-suppressed plants versus control nontransgenic ones: Whereas the lesions on the control plants remained restricted in size, from days 6 to 8 after inoculation those on LeCHRc-suppressed plants spread, covering up to 60% more area (versus controls) 8 d postinoculation. Mock-inoculated plants did not generate lesions on either stems or leaves. Moreover, tomato transgenic lines characterized by Vishnevetzky et al. (1999b) that overaccumulate CHRc were significantly less susceptible to B. cinerea infection, as compared to both control nontransgenic plants and LeCHRc-suppressed plants (Table III), further indicating the involvement of LeCHRc in the plant’s resistance to the fungus. As expected, following inoculation with B. cinerea, no expression of LeCHRc was detected in RNAi-transgenic plants, whereas in control plants LeCHRc levels increased (Fig. 8B).

**DISCUSSION**

From evolutionary and phylogenetic standpoints, PAPs appear to be a very old and conserved group of genes (Vishnevetzky et al., 1999a). Their role in sequestering overaccumulated carotenoids within chloroplasts of angiosperm reproductive organs may be a rather recent development, whereas their original function may have been to aid in the sequestration of hydrophobic molecules and protect against various stresses. Indeed, PAPs are up-regulated by various stresses, e.g. drought, mechanical wounding, treatments generating active oxygen species, and biotic stresses (Pruvot et al., 1996; Chen et al., 1998; Gillet et al., 1998; Manac’h and Kuntz, 1999; Langenkamper et al., 2001). Numerous stress conditions, such as heat shock at 42°C and inoculation with different viruses (Tomato mosaic virus, Potato virus Y, and Tomato yellow leaf curl virus) and fungi (Oidium sp. and B. cinerea), resulted in the induction of ChrC and LeCHRc in leaves of cucumber and tomato, respectively (Fig. 5B; data not shown). Using ChrC promoter fused to GUS, we further showed (Fig. 5) that similar to Fib, activation by both biotic and abiotic stresses occurs at the transcriptional level. Monte et al. (1999) and Rey et al. (2000) have shown that suppression of the PAP CDSP34 leads to increased sensitivity of potato to photooxidative stress, and overexpression of Fib in tobacco improves plant performance under high-light conditions. Oxidative events are also strongly implicated in the interaction of B. cinerea with plants (Malolepsza, 2005). This pathogen has a broad host range, causing gray mold on more than 200 plant species, including tomato (Elad et al., 2004). Using a tomato plant system, we show that LeCHRc expression is needed for resistance to B. cinerea infection. Transgenic plants with suppressed LeCHRc were significantly more susceptible to infection in both in vitro experiments with detached leaves and in the growth chamber with intact leaves and stems (Tables II and III).

The production of active oxygen species during infection of plants by B. cinerea has been well documented, and there is growing evidence that the fungus exploits this situation to aid its development in planta (Lyon et al., 2004). There is now evidence (Urbanek et al., 1996; Von Tiedemann, 1997) that the generation of active oxygen species assists in the colonization of plant tissues by necrotrophic organisms, such as B. cinerea. One of the most obvious and consistent effects of B. cinerea infection is a large increase in the electron paramagnetic resonance free-radical signal in rotted tissue compared to controls; similar results have been obtained with leaves and fruit (Deighton et al., 1999; Muckenschnabel et al., 2001). LeCHRc, similar to other PAPs, may assist in contending with the oxidative stress: Its suppression may lower the plant’s ability to withstand the oxidative stress associated with B. cinerea attack, hence leading to increased susceptibility to infection.

The involvement of LeCHRc in resistance to biotic stresses further supports the hypothesized general, evolutionarily preserved role of PAPs in plant protection. Conservation of PAPs is also evident from the fact

**Table I.** Carotenoid levels in transgenic tomato with suppressed LeCHRc expression versus control plants

<table>
<thead>
<tr>
<th>Carotenoid in Corollas</th>
<th>Carotenoid in Leaves</th>
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<tbody>
<tr>
<td>ng/μg protein</td>
<td>ng/μg protein</td>
</tr>
<tr>
<td>Line</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>11</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>37</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>13</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>19</td>
<td>5.8 ± 0.2</td>
</tr>
<tr>
<td>WT</td>
<td>6.0 ± 0.3</td>
</tr>
</tbody>
</table>

**Table II.** Susceptibility of detached transgenic tomato leaves with modulated ChrC expression levels to B. cinerea infection

<table>
<thead>
<tr>
<th>Line</th>
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<th>6 d</th>
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<tbody>
<tr>
<td>Sense</td>
<td>0.6 c</td>
<td>3.6 c</td>
</tr>
<tr>
<td>11</td>
<td>1.6 b</td>
<td>19.0 a</td>
</tr>
<tr>
<td>13</td>
<td>1.2 b</td>
<td>15.0 a</td>
</tr>
<tr>
<td>37</td>
<td>1.9 a</td>
<td>19.0 a</td>
</tr>
<tr>
<td>WT</td>
<td>0.9 c</td>
<td>6.5 b</td>
</tr>
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that even the sequence of a *Synechocystis* homolog shares approximately 30% identity with plant PAPs (Vishnevetsky et al., 1999a). It is reasonable to assume that during their evolution, PAPs were harnessed for diverse cellular activities. Originally, PAPs were proposed to play a role in the organization of carotenoid-accumulating structures in flower and fruit chloroplasts. This was mainly based on their intrachloroplast location and on the correlation between their expression pattern and carotenoid levels in chloroplasts during flower/fruit development (Deruere et al., 1994; Vishnevetsky et al., 1999a). Here, we further evaluated the role of CHRC in carotenoid accumulation within chloroplasts in floral tissues, using transgenic tomato plants with suppressed *LeCHRC*. Analyses of carotenoid levels in flowers with down-regulated *LeCHRC* relative to controls revealed that the former accumulate up to 32% less carotenoids per unit protein, strengthening the notion of an interrelationship between PAPs and flower-specific carotenoid accumulation in chloroplasts.

The functional characterization of PAPs by RNAi is strongly hindered by the presence in plastids of several members of this family, as has been shown in various plant systems (Laizet et al., 2004; Murphy, 2004; Ytterberg et al., 2006). In Arabidopsis, for example, 13 genes encoding members of this family have recently been identified, some of which appear to be the result of recent gene duplication (Laizet et al., 2004). The functional divergence of PAPs is also evidenced by their different patterns of expression, as revealed by both proteome and transcriptome analyses, in chloroplastogenic versus nonchloroplastogenic tissue (Laizet et al., 2004; Murphy, 2004; Ytterberg et al., 2006). A search in the National Center for Biotechnology Information and TIGR databases for PAPs reveals only one contig in cucumber and two homologs (TC162898 and TC161992, the latter characterized here) in tomato. The homology between the two tomato open reading frames is low: 44.5% at the nucleotide level with 37% identity at the amino acid level. The *LeChrC* characterized here is 95.7% identical in amino acids with *Fib*, whereas the second homolog (TC162898) is identical in only 37.4% of its amino acids. Clearly, we cannot exclude the occurrence of additional PAP homologs with redundant or overlapping functions, as no genome sequence is currently available for the plant species studied here. To evaluate the role of *LeCHRC*, we applied an RNAi approach using a nonconserved 5′ gene region. This in itself does not ensure specificity of suppression for individual family members (Valencia-Sanchez et al., 2006). However, since only two *LeChrC* homologs were identified in the databases and the fragment used for RNAi was generated from a divergent region, and since the promoter of *ChrC* is responsive to both chloroplastogenesis and stress signals, we are tempted to propose

<table>
<thead>
<tr>
<th>Line</th>
<th>Disease Severity on Leaves</th>
<th>Lesion Size on Stem</th>
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<tr>
<td></td>
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<td>6 d</td>
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<tr>
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<td>21.5 c</td>
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<td>3.4 b</td>
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<td>37</td>
<td>5.0 b</td>
<td>86.8 a</td>
</tr>
<tr>
<td>WT</td>
<td>4.6 b</td>
<td>54.8 b</td>
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</tbody>
</table>

Table III. Susceptibility of transgenic tomato plants with modulated ChrC expression levels to *B. cinerea* infection

Transgenic tomato overexpressing ChrC (sense), control nontransgenic (WT), and RNAi-suppressed plants (independent lines 11, 13, and 37) were infected with *B. cinerea*. Disease severity on leaves, measured 3 and 6 d after inoculation, was determined by estimating the size of the necrotic area on a scale of 0% to 100%, where 100% severity = a lesion of 20 mm². Stem infection, 6 and 8 d after inoculation, was evaluated by measuring the length of the lesion. Numbers followed by a common letter are not significantly different (*t* test, *P* ≤ 0.05).
that the characterized phenotype is due to \textit{LeChrC} suppression.

Regulation at the RNA level has been shown to control PAPs in diverse processes/tissues, i.e. developmental and hormonal expression and induction by biotic and abiotic stresses of both \textit{ChrC} and \textit{Fib} (Fig. 5; Kuntz et al., 1998; Vishnevetsky et al., 1999b). While carotenoid type/amount exerts a posttranscriptional effect on \textit{ChrC} expression, isoprenoid pathway-related, flower-specific trans-factors were proposed to modify its expression at the transcriptional level (Vishnevetsky et al., 1999b). The MYBYS characterized here may represent such a \textit{ChrC}-activating trans-factor. Its expression is spatially controlled with no detectable levels in leaves. In floral tissue, it is temporally regulated, mimicking the increase in \textit{ChrC} levels during flower development (Fig. 1B). The ability of MYBYS to specifically trans-activate \textit{ChrC} promoter was shown by two alternative approaches (Fig. 2). Ectopic expression of MYBYS was sufficient for activation of \textit{ChrC} promoter in young green tomato flowers. Using co-bombardment of \textit{ChrC}:GUS and 35S:MYBYS, GUS expression was also revealed in floral tissues of different plants that accumulate anthocyanin and not carotenoids, such as petunia and carnation. No GUS expression was observed when MYBYS or \textit{ChrC} promoter was replaced with other transcription factors/promoters. In leaves, no trans-activation of \textit{ChrC} promoter was observed, indicating that MYBYS is not sufficient to activate \textit{ChrC} promoter in tissues other than flowers. It should be noted that the ability of MYBYS to specifically activate \textit{ChrC} promoter was recently harnessed to develop a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants (Tzfira et al., 2005).

In addition to isoprenoid pathway-related trans-factor MYBYS, also GA$_3$, a product of the pathway, has been shown to rapidly activate \textit{ChrC} expression in floral tissues in parallel to chromoplastogenesis. Activation of \textit{ChrC} by GA, localized to a 290-bp region of the promoter, was characterized as a primary response (Vishnevetsky et al., 1997, 1999b), in contrast to some other well-established, GA-regulated systems, such as the flavonoid pathway in flowers and activation of the \textit{α}-amylase gene in aleurone cells, which are slow-response processes that depend on de novo protein synthesis (Weiss et al., 1992; Huttly and Phillips, 1995).

A detailed characterization of mainly GA-responsive mutants in various plants allowed the identification of DELLA proteins functioning as negative regulators in the GA-signaling cascade (Gomi and Matsuoka, 2003). In barley (\textit{Hordeum vulgare}), GAMyb, a transcriptional activator of \textit{α}-amylase gene expression that is rapidly regulated by GA, was shown to be the target, although not necessarily a direct one, for this class of proteins (Gubler et al., 2002). However, cis-elements responsible for its GA responsiveness, in contrast to GARE/CARE elements of the slow-response \textit{α}-amylase genes, were not characterized (Gubler et al., 1999; Sutoh and Yamauchi, 2003). Dissection of 290 bp of the \textit{ChrC} promoter revealed an 18-bp motif that was necessary for the GA responsiveness. Further scanning of this motif allowed the identification of three nucleotides (gaCTCc), the mutation of which abolished GA responsiveness. Interestingly, this new motif is partially similar to the GA-responsive CARE regulatory element (CAACTC) in the promoters of rice (\textit{Oryza sativa}) and barley Cys proteinases (REP-1 and EPB1) and the rice \textit{α}-amylase gene (RAMy1A; Sutoh and Yamauchi, 2003). Deletion of the 18 bp or mutation of three nucleotides in the GA-responsive motif of the \textit{ChrC} promoter (Figs. 3 and 4) led to its increased activity, to a level similar to that of the native promoter following GA activation. This suggests that negative regulation, similar to other GA-responsive systems, underlies the response of \textit{ChrC} to GA. The observation that responsiveness of the promoter to GA and MYBYS, both related to the isoprenoid pathway, does not overlap with respect to cis-elements brings additional support to the notion that throughout evolution, plants have integrated diverse functions into PAPs. To this end, their involvement in resistance to stresses/diseases further emphasizes the complexity of the machinery governing the multitasking of plant components.

MATERIALS AND METHODS

Plant Material and in Vitro Flower Bud Culture

Cucumber (\textit{Cucumis sativus} L. cv Shimson) and tomato (\textit{Lycopersicon esculentum} Mill. cv Adi) plants were grown under standard greenhouse conditions (Vishnevetsky et al., 1999b). Flower development was divided into stages: In cucumber, stages 1, 2, and 3 represent flowers 120, 72, and 24 h before anthesis, respectively, and stage 4 flowers are at anthesis; in tomato, stages 1, 2, and 3 represent flowers 84, 48, and 12 h before anthesis, respectively, and stage 4 flowers being at anthesis.

In vitro culture of flower buds was performed as described previously (Vishnevetsky et al., 1997). Stage 1 flower buds were cultured in double distilled water for 12 h and then transferred to fresh double distilled water or 100 μM GA$_3$ (Sigma-Aldrich) for another 12 h prior to bombardment.

Isolation of the MYBYS Regulatory Factor

A Zap-cDNA expression library from stage 3 flower corollas (Vishnevetsky et al., 1996) was screened with radiolabeled promoter region of \textit{ChrC} (137-bp fragment, positions −215 to −78) to identify cDNA clones encoding \textit{ChrC}-DNA-binding protein. Approximately 10$^5$ bacteriophages were screened in the presence of an excess of nonspecific competitor calf thymus DNA (Ausbel et al., 2001), and, following two rounds of rescreening, potential recombinants of interest were isolated and sequenced.

Constructs for Transient Expression and Particle Bombardment

The \textit{mybys} gene was introduced into a PCD vector (Broido et al., 1991) 3' to the CaMV 35S promoter, to generate 35S:MYBYS plasmid. The ChrC:GUS construct (pGEM2Z/201.2; Vishnevetsky et al., 1999b), containing 3,500 bp of \textit{ChrC} promoter upstream of the GUS gene, was digested with EcoRI and EheI to generate the deletion within the \textit{ChrC} promoter region between positions −290 and −78 (Δ212ChrC:GUS). The 212-bp fragment (position −290 to −78) released from the \textit{ChrC} promoter region was introduced 5' to a minimal (−46 to +8) TATA-box promoter fused to a GUS reporter gene, creating 212ChrC:GUS. This plasmid was used in all further \textit{ChrC} promoter 5' deletions. Plasmid containing a promoter fragment (−215 to −78) fused to TATA:GUS (137ChrC:GUS) was generated following digestion of 212ChrC:
GUS with PnuCl and Apal and blunt ligation. To generate plasmids containing promoter fragments –141 to –78 and –124 to –78 fused to TATA-GUS, PCR fragments of the relevant promoter regions were used. Primers used for the generation of the former fragment were F-63 (5'-GACCTCCAAACACA-GACA-3') and R (5'-TCACGGGTGGGTTGTTCATAC-3'), and for the latter fragment primers were F-46 (5'-CAAGTTCCAGAAGAAGAGC-3') and R. Mutagenesis of the four adjacent regions (six nucleotides each) within the –141 to –124 promoter fragment was generated using primers containing a GTATC replacement of the promoter sequence: MG1 (5'-GATCTACAAACACA-GACAATTTTCGGA-3'), MG2 (5'-CTGATATCAACGGAAGTTCGGA-3'), MG3 (5'-CTCTACATGTAGAAGTGTCGGA-3'), and MG4 (5'-GACCTCCAAACATGTAATTTTCGGA-3').

Transient expression of the constructs in leaves and petals was evaluated following bombardment using the Biolistic PDS 1000/He system (Bio-Rad) at a pressure of 1.350 dpsi (effector to reporter taken in 1.5 molar ratio), as described by Vishnevetsky et al. (1999b). In MYBS transient-expression experiments, the transcriptional regulator Pap1 (production of anthocyanin pigment) 1 fused to CaMV 35S (35S:PAP vector; Ben-Meir, 2003) was used as a control MYB factor. Following bombardment, tissue was incubated for a few hours at 37°C in a 0.1% (w/v) X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide; Duchefa) solution containing 0.1% sodium phosphate buffer, pH 7.0, 10 mM EDTA, and 0.1% (w/v) Triton X-100 (Vishnevetsky, 1999). In GA experiments, EGFP driven by CaMV 35S (pEGFP-PL vector; Ben-Nissan et al., 2004) was co-bombarded as reference, and GFP expression was monitored using a fluorescence binocular (480/40 nm excitation filter and 510 nm barrier filter; MZ FLIII; Leica) equipped with a DC300FX camera (Leica), prior to transfer of the tissue to the X-Gluc solution. GUS expression was normalized to GFP using ImageJ software (Bezanilla et al., 2003). All experiments were repeated at least five times.

LeChrC and MYBS Vectors and Tomato Transformation

Based on the tomato EST database, the ChrC homolog was identified (Tc9807) and cloned (LeChrC, 775 bp) using two oligonucleotide primers: F47 (5'-TGCTCTTTCTCTGTTTCACTCTGA-3') and R822 (5'-TTGTCCTCCAAAGAATITCACGTC-3'). A 5' LeChrC fragment (530 bp) generated using PCR primers 5'-ATGCTCTACCTTCTCTCTCTCA-3' and 5'-TCGACAGCAGACGCAGATGTC-3' was cloned into pRNA69 (Waterhouse et al., 1998) 3' to the CaMV 35S promoter in an antisense and sense orientation before and after the intron, respectively. The resultant plasmid was digested with NolI, and the fragment was inserted into the binary vector pART27 to create the RNAi construct (Gleave, 1992). For the MYBS sense construct, 35S:MYBS was released from 35S:MYBS plasmid using BamHI and XhoI and transferred to the binary vector pCNG559 (Comai et al., 1990). Binary vectors were electroporated into Agrobacterium tumefaciens strain AGL0 and used for transformation of tomato cv 'Adami' as described by Vishnevetsky et al. (1999b).

RNA-Blot, RT-PCR, and Western-Blot Analyses

Total RNA, isolated as described previously (Vishnevetsky et al., 1996), was used in either blot or RT-PCR analysis. For the former, 10 μg of total RNA was fractionated through a 1.6% formaldehyde gel and transferred to a Hybond-N+ filter (Amersham Biosciences). cDNA clones of myb (371–885 nucleotide fragment, upstream of the conserved R2/R3 DNA-binding sites), ChrC, and LeChrC served as specific probes following labeling with 32P using a random priming kit (Rediprime; Amersham Biosciences). The blots were hybridized as described by Vishnevetsky et al. (1996). Hybridization was at 60°C, and the membranes were washed twice in 2 X SSC, 0.1% (w/v) SDS at 60°C for 20 min each and exposed to x-ray film (Fuji) at ~70°C. For RT-PCR, total RNA was treated with RNase-free DNase (Promega) and transcribed using oligo(dT)12 primer (Promega) and M-MLV reverse transcriptase (Promega) according to the manufacturer’s recommendations. For PCR amplification of LeChrC, primers F47 and R822 were used. The reaction was performed in the presence of an additional two primers, 5'-GGTTTTGCCTGGGATGTC-3' and 5'-CAATGAGATICTCTCAACAGATTGAGTGCTC-3', allowing amplification of reference actin cDNA.

Proteins were extracted from stage 3 tomato cotyledons, fractionated by 12.5% SDS-PAGE (50 μg per lane), and analyzed following western blotting using affinity-purified polyclonal antibodies against CHR and a chimerimun- cence detection kit (Amersham Biosciences; Vishnevetsky et al., 1999b). Membranes were then exposed to x-ray film (Fuji). Prior to incubation with antibodies, membrane was stained with Poncze-S red (Sigma-Aldrich).

Botrytis Infection and Measurements

Botrytis cinerea (isolate Bc116; Guetsky et al., 2002) was maintained and grown for infection experiments on potato dextrose agar. Conidia were harvested from the cultures by agitating small pieces of 14-d-old agar bearing mycelium and conidia in a glass tube containing 2 mL of tap water and 0.01% (w/v) Tween-80. The suspension was filtered through a double layer of cheesecloth to screen out mycelium plugs, and the conidial concentration was calibrated by means of a hemacytometer and adjusted to 5 × 106 cells/mL. Glc (0.05%, w/v) and KH2PO4 (0.05%, w/v) were added to the conidial suspension. Detached leaves were infected by placing 8 to 20 μL drops of the suspension on each of six leaves from each plant line. The leaves were then placed on a plastic grid that was laid over moist paper, all of which was then placed in a box covered with transparent polyethylene to ensure high humidity. In experiments with whole plants, four leaflets of each of four leaves on six plant replicates (1-month-old tomato plants) were infected with 20 μL of conidial suspension and covered with a polyethylene bag. Care was taken to avoid contact between the polyethylene and the inoculum drop. Stem infection was carried out with 5 mm-diameter mycelium discs that originated from the edge of a 4-d-old potato dextrose agar culture of B. cinerea. The mycelium disc was placed on the stem surface between the second and third nodes. Leaves and plants were incubated in a walk-in growth chamber set at 20°C ± 2°C and a 12-h photoperiod.

Disease severity on leaves in all experiments was determined by estimating the size of the necrotic area developed from each suspension drop on a scale of 0% to 100%, where 100% severity = a lesion of 20 mm² (Guetsky et al., 2002). Severity of stem infection was evaluated by measuring the length of the lesion.

Analytical Methods

Carotenoid contents were determined as described by Lichtenthaler (1987). Protein content was determined using a detergent-compatible protein assay (Bio-Rad).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ311672 and DQ310151.

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LITERATURE CITED


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