CHRC, Encoding a Chromoplast-specific Carotenoid-associated Protein, Is an Early Gibberellic Acid-responsive Gene*

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CHRC, a corolla-specific carotenoid-associated protein, is a major component of carotenoid-lipoprotein complexes in Cucumis sativus chromoplasts. Using an in vitro flower bud culture system that mimics in vivo flower development, CHRC mRNA levels in corollas were shown to be specifically up-regulated by gibberellic acid. The response to gibberellic acid was very rapid (within 20 min) and insensitive to protein synthesis inhibition by cycloheximide. Abscisic acid, known to antagonize gibberellin in many developmental systems, strongly down-regulated CHRC mRNA levels. The gibberellin synthesis inhibitor paclobutrazol exhibited a similar negative effect on CHRC expression. Inclusion of exogenous gibberellic acid into the in vitro bud culture system with the paclobutrazol not only prevented the CHRC mRNA down-regulation, it up-regulated transcript accumulation to the level of gibberellic acid-treated corollas. CHRC mRNA accumulation in response to gibberellic acid displayed a dose-dependent increase up to 10⁻⁴ M gibberellic acid. The up-regulation could be detected with as little as 10⁻⁷ M gibberellic acid. Based on these data, we suggest that CHRC is the first structural gene identified to date whose expression is regulated by gibberellic acid in a primary fashion. The critical role of the rapid response of CHRC to gibberellic acid in aiding carotenoid sequestration while preserving chromoplast structural organization is discussed.

Gibberellins (GAs)¹ are plant hormones that regulate various aspects of plant growth and development, such as germination, cell growth, stem elongation, flower and fruit development, and pigmentation. Despite their critical role, understanding the molecular mechanisms of the action of GAs remains one of the major challenges in plant biology. Although it is now well established that GAs modulate gene expression (1, 2), no causal relationship between a particular growth process and GA-mediated changes in gene expression has been established. It is still unknown whether responses of different genes to GAs are mediated by a single or number of pathways (1–3).

In recent years, information has begun to accumulate on the molecular events involved in conveying the signal of GA from the as yet to be identified receptor, through the cytoplasm to the nucleus (1–6). The aleurone layer of cereal grains is probably the best characterized system with respect to molecular mechanisms involved in GA-regulated gene expression. This is a slow response system that depends on de novo protein synthesis to activate gene expression of hydrolytic enzymes (1, 2, 7). The plasma membrane is the most probable site of GA perception, and calcium/calmodulin and GMP have been implicated in early events of signal transduction (4–6). The factors regulating these events and linking them with the modulation of gene expression are unknown.

An elucidation of the primary mechanism(s) of GA action could provide clues to understanding the signal transduction cascade(s) leading from the perception of GA to altered gene expression. Per definition, the induction of primary response genes is independent of de novo protein synthesis (8). This implies that the components required for the transcriptional activation of such genes are pre-existing and that the extracellular stimulus is transduced to the nucleus via post-translational processes. Consequently, the number of intervening steps is limited, and the time elapsing between ligand perception and gene activation is short, usually on the order of minutes to a few hours. Very little is known about such primary genes, which are responsive to GAs. Although a number of genes or their products have shown GA-dependent up-regulation in a short period of time (2, 9–11), only two of them, both from the family of Myb transcription regulators, exhibit primary responses. GAmyb from barley aleurone cells is up-regulated by gibberellic acid (GA₃) in 3 h and has been found to be a transcriptional activator of high pi α-amylase gene promoter (10); pmyb92 from petunia corollas, of unknown function, is up-regulated by GA₃ within 30 min (11).

The involvement of GAs in various aspects of cucumber (Cucumis sativus L.) floral organ development is well documented and has been put to practical use in both the field and greenhouses (12). Recently, the involvement of GA₃ in chromoplast biogenesis in corollas was characterized. Two chromoplast-specific carotenoid-associated proteins (CHRC and CHRD) were identified and shown to be specifically up-regulated by GA₃ (13–15). Whereas carotenoid levels due to GA₃ application increased slowly (detectable after 6 h), the CHRC and CHRD protein levels were rapidly enhanced. The specific up-regulation of these proteins by GA₃ was detectable within 2 h, and abscisic acid (ABA) and paclobutrazol counteracted this effect (14, 15). Isolation and characterization of a single-copy gene coding for CHRC (16) allowed us to detail its regulation by GA₃.

Here we report strong evidence of the primary response of CHRC to GA₃. To the best of our knowledge, CHRC is the first reported structural gene whose expression is up-regulated by GA₃ in a primary fashion. Its up-regulation at the mRNA level could be detected within 20 min of GA₃ treatment. Paclobutrazol, a GA synthesis inhibitor, and ABA strongly down-regulated CHRC mRNA levels, whereas the protein synthesis inhibitor cycloheximide (CHX) did not interfere with the up-

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¹ The abbreviations used are: GA, gibberellin; GA₃, gibberellic acid; ABA, abscisic acid; CHX, cycloheximide; PSY, phytoene synthase.
regulation of CHRC by GA$_3$. A crucial role for GAs during flower development, chromoplastogenesis, and carotenoid accumulation in *C. sativus* is suggested.

**EXPERIMENTAL PROCEDURES**

*Plant Material and in Vitro Flower Bud Culture—C. sativus* L. plants (cultivar Shimshon, obtained from Zeraim Gedera, Israel) were grown under standard greenhouse conditions. In *vitro* culture of flower buds was performed at 23 °C, under constant light from cool white fluorescent lamps with a photosynthetic photon flux density of 80 mol m$^{-2}$ s$^{-1}$, as described previously (14). Briefly, buds at developmental stage 1 (~8 mm in length, 120 h before anthesis) were collected and rinsed several times with sterile water. Buds were then placed for 24 h on a perforated Parafilm (American National Can, Greenwich, CT) covering a Petri dish filled with double-distilled sterile water, such that only the bases of the buds were in contact with the liquid. Following equilibration, buds were transferred to a fresh Petri dish (zero time for the experiments) and treated with GA$_3$, ABA, paclobutrazol, or CHX, as specified in the figure legends.

RNA Isolation and Northern Blot Analysis—Total RNA from corollas of cucumber flower buds was isolated as described previously (16). RNA (15 and 25 μg) was fractionated through a 1.2% formaldehyde gel and transferred to GeneScreen$^{	ext{TM}}$ + N$^{	ext{TM}}$ + Plus nylon membranes (Du Pont, NEN Division). A random priming kit (Boehringer Mannheim) was used to radioactively label DNA probes. The blots were hybridized with $^{32}$P-labeled cucumber CHRC cDNA (16) and reprobed with melon phytoene synthase (PSY) cDNA (MEL5) (18), kindly provided by Prof. D. Grierson (Nottingham University, UK). The hybridization for analysis of CHRC expression was carried out in 0.263 M Na$_2$HPO$_4$, 7% SDS, 1 mM EDTA, 1% BSA for 16 h at 60 °C, and the washes were performed in 2 × SSC/0.1% SDS at 50 °C followed by 2 × SSC/0.1% SDS at 55 °C, for 20 min each. Hybridization with the MEL5 probe was carried out at 55 °C, using the same procedure. Following the hybridization, the blots were washed in 5 × SSC/0.1% SDS at 45 °C for 20 min followed by 5 × SSC/0.1% SDS at 50 °C and 2 × SSC/0.1% SDS at 50 °C for 20 min each. Autoradiograms were quantified by scanning suitably exposed films in a densitometer (Molecular Dynamics, Sunnyvale, CA). The amount of RNA loaded onto the gels was standardized by optical measurement, by quantitation of the ethidium bromide fluorescence of cytoplasmic RNA, and by the level of hybridization with a DNA fragment coding for cytoplasmic 18S RNA (19).

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—SDS-polyacrylamide gel electrophoresis of the total corolla proteins (10, 25, and 50 μg/lane) and Western blot analysis using affinity-purified polyclonal antibodies against CHRC and an enhanced chemiluminescence system (Amersham Corp.) were performed as described previously (14). Autoradiograms were quantified by scanning suitably exposed films in a densitometer (Molecular Dynamics).

**RESULTS**

Rapidity and Specificity of the GA$_3$ Response—An in *vitro* flower bud culture system, previously shown to mimic in *vitro* flower development (13), was used to study the effect of GA$_3$ on CHRC expression. CHRC mRNA level was very rapidly affected by exogenous GA$_3$ (Fig. 1A); after 20 min of treatment it was approximately twice as high in treated versus control corollas. With longer incubation periods, the level of CHRC mRNA in GA$_3$-treated corollas increased further, to approximately 5- to 6-fold that of untreated control corollas (Fig. 1). A closer examination of early time points revealed a detectable increase in CHRC mRNA level within 10–20 min in response to GA$_3$ (data not shown). At the protein level, the effect of GA$_3$ on CHRC was slower; after 2 h of treatment, ~2.5 times more CHRC per unit of protein had accumulated in treated versus control corollas (Fig. 1A). No changes in the total protein content of corollas were detected within 6 h of treatment. To determine whether GA$_3$ also affects the expression of genes encoding enzymes from the carotenoid biosynthesis pathway, the expression of *Psy*, the first committed gene of that pathway, was studied. *Psy* mRNA levels were rather stable during the first 48 h of corolla development and were not affected by GA$_3$ treatment (Fig. 1).

To further characterize the involvement of GAs in CHRC expression, the effect of paclobutrazol, an inhibitor of GA synthesis, on CHRC mRNA levels was analyzed (Fig. 2). The addition of paclobutrazol to the *in vitro* bud culture system led to a 4-fold decrease in CHRC mRNA levels relative to control untreated corollas. Inclusion of exogenous GA$_3$, in addition to paclobutrazol not only prevented the down-regulation, it up-regulated CHRC transcript accumulation to the level of GA$_3$-treated corollas (Fig. 2).

ABA antagonizes GA in many developmental processes and has been shown to have an inhibitory effect on CHRC protein accumulation (15). To obtain additional support for the role of GA$_3$ in the regulation of CHRC expression, the effect of ABA on CHRC transcript accumulation was analyzed. When 100 μM ABA was added to the *in vitro* bud culture system, the CHRC mRNA level was down-regulated 2.5 times as compared with control corollas (Fig. 2). The inhibitory effect of ABA on CHRC transcript accumulation was concentration-dependent. Application of 10 μM ABA only slightly down-regulated CHRC expression (data not shown). Neither ABA nor paclobutrazol markedly affected *Psy* mRNA levels (Fig. 2).

Effect of the Protein Synthesis Inhibitor CHX—To study the effect of protein synthesis inhibition on the regulation of CHRC by GA$_3$, cucumber flower buds could be cultured with or without CHX in the presence of GA$_3$. As can be seen from Fig. 3, 50 μM CHX did not prevent the up-regulation of CHRC mRNA by GA$_3$, whereas it successfully abolished the increase in CHRC protein levels in GA$_3$-treated buds. CHX alone did not affect CHRC transcript accumulation (Fig. 3), in contrast to its up-regulating effect on the early response *GAMYB* gene in barley aleurone cells (10). *Psy* mRNA levels in corollas were also unaffected by CHX treatment (Fig. 3).

Dose Response to GA$_3$—The sensitivity of CHRC expression to GA$_3$ was assessed. A dose response curve ranging from $1 \times 10^{-8}$ to $1 \times 10^{-4}$ μM GA$_3$ was obtained after exposing *in vitro* grown flower buds to the GA$_3$ for 2 h (Fig. 4). CHRC was strongly up-regulated at $10^{-5}$ to $10^{-4}$ μM, whereas concentrations as low as $10^{-7}$ μM were sufficient to markedly elevate basal CHRC mRNA levels (Fig. 4). A similar response curve has been...
CULTURED FOR 6 HINT THE PRESENCE OF 100 RNA AND PROTEIN WERE EXTRACTED FROM CUCUMBER COROLLAS OF FLOWER BUDS BEEN USED TO CHARACTERIZE MANY GA-REGULATED GENES. TO DATE, BEEN ESTABLISHED TO STUDY GA ACTION (1–3). THESE SYSTEMS HAVE PROVEN TO BE SLOW RESPONSE GENES AND HENCE DO NOT ALLOW EASY ACCESS TO A STUDY OF THE INITIAL STEPS OF GA ACTION. EXPERIMENTAL SYSTEMS RESPONDING TO GA₃ IN A PRIMARY FASHION COULD BE VERY HELPFUL IN DELINEATING THESE STEPS. PRIMARY RESPONSE SYSTEMS ARE CHARACTERIZED BY A FEW LIMITING, ALbeit USEFUL CRITERIA; THE EFFECT ON GENE EXPRESSION SHOULD BE SPECIFIC, RAPID, AND UNAFFECtED BY PROTEIN SYNTHESIS INHIBITORS (8, 20). IN THE PRESENT STUDY, WE SHOW SPECIFIC UP-REGULATION OF CHRC EXPRESSION IN CUCUMBER COROLLAS BY GA₃; PACLOBUTRAZOL AND ABA DOWN-REGULATED ITS EXPRESSION, WHEREAS PSY EXPRESSION WAS NOT AFFECTED. THE UP-REGULATION OF CHRC mRNA LEVEL BY GA₃ WAS VERY RAPID, OCCURRING WITHIN 20 MIN. THIS RESPONSE IS CONSIDERABLY FASTER THAN THE WELL STUDIED GA3 INDUCTION OF α-AMYLASE mRNA IN ALEURONE CELLS AND FLAVONOID GENE ACTIVATION IN PETUNIA FLOWERS (1, 2, 7, 21) AND IS COMPARABLE IN KINETICS TO THE RESPONSE OF MOST RAPIDLY AUXIN-REGULATED PRIMARY RESPONSE GENES (22). THE VERY SHORT LAG PERIOD BETWEEN THE ADDITION OF GA₃ AND THE ACCUMULATION OF CHRC TRANSCRIPT SUGGESTS PRIMARY HORMONE ACTION. THE ABSENCE OF AN INHIBITORY EFFECT BY CHX ON GA₃ UP-REGULATION OF CHRC mRNA LEVELS FURTHER SUPPORTS THIS SUGGESTION.

CHX HAS BEEN USED IN VARIOUS SYSTEMS TO EXAMINE WHETHER REGULATION OF GENE EXPRESSION requires SUSTAINED PROTEIN SYNTHESIS. Interestingly, in some early inducible systems, including GAMYB, SAUR, and Aux/IAA genes, the simultaneous presence of CHX and the inducer resulted in superinduction of the respective mRNAs (10, 22). In these systems, CHX could be exerting a dual effect as an inducer of transcription and a stabilizer of the inducible mRNA. In other cases, such as the auxin-inducible GH3 gene family or the GH1 gene from soybean (22), as well as that of CHRC presented here, CHX alone does not up-regulate gene expression.

THE RELATIONSHIP BETWEEN GAs AND CHRC GENE EXPRESSION IS NOT SURPRISING. TISSUE/ORGAN-SPECIFIC CAROTENOID-DERIVED PIGMENTATION AS WELL AS ANTHOCYANIN-DERIVED PIGMENTATION IS KNOWN TO BE UNDER TIGHT GA CONTROL (14, 15, 21, 23–26). GAs AS WELL AS CAROTENOIDs ARE ISOPRENOIDS FORMED BY A COMMON PATHWAY ORIGINATING FROM THE CONDENSATION OF ISOPRENE UNITS (27). THE FIRST COMMITTED STEP IN CAROTENOID BIOSYNTHESIS IS THE CONVERSION OF TWO MOLECULES OF GERANYL-GERANYL DIPHOSPHATE INTO PHYTOENE CATALYZED BY PSY. GAs ARE ALSO DERIVED FROM GERANYLGERANYL DIPHOSPHATE THROUGH CYCLIZATION OF ENT-KAURENe. SYNTHESIS OF THE LATTER, AS WELL AS OF CAROTENOIDS, OCCURS IN PLASTIDS. RECENTLY, INHIBITION OF PSY BY AN ANTISENSe TRANSGene WAs SHOWN TO RESULT IN THE ACCUMULATION OF INCREASED GA LEVELS IN IMMATURE TOMATO FRUITS (25). Moreover, overexpression of PSY led to a reduction in GAs concentration (24). In the present study, PSY mRNA Levels were not affected by GA3 application. Thus, modulation of carotenogenesis by GA3 is most probably executed via regulation of downstream carotenoid biosynthesis enzymes and/or carotenoid sequestration. CHRC AND SIMILAR PROTEINS IN CHROMOPLAST-CONTAINING TISSUES ARE ESSENTIAL STRUCTURAL COMPONENTS OF LIPOPROTEIN COMPLEXES (26). BECAUSE CAROTENOID-ASSOCIATED CHRC IS A MAJOR PROTEIN IN THESE COMPLEXES, ITS RAPID UP-REGULATION IN RESPONSE TO GA₃ IS PROPOSED TO BE CRUCIAL FOR ENHANCED CAROTENOID ACCUMULATION WHILE PRESERVING THE STRUCTURAL ORGANIZATION OF THE CHROMOPLAST, HENCE THE TIGHT RELATIONSHIP BETWEEN GAs AND CHRC. THE NEGATIVE EFFECT OF THE GA BIOSYNTHESIS INHIBITOR PACLOBUTRAZOL ON CHRC TRANSCRIPT ACCUMULATION, THE ABILITY OF EXOGENOUS GA₃ TO ABOLISH THIS EFFECT, AND THE DOWN-REGULATION OF CHRC BY ABA FURTHER IMPLICATE THE INVOLVEMENT OF ENDOGENOUS GAs IN CHRC REGULATION.

RESULTS PRESENTED HERE STRONGLY SUPPORT THE CRITICAL ROLE OF GA IN CHROMOPLASTOGENESIS, EXECUTED VIA THE MODULATION OF SPECIFIC FLOWER GENE EXPRESSIONS. WE PRESENT EVIDENCE THAT THE CHRC GENE FROM C. SATIVUS, ENCODING A CHROMOPLAST-SPECIFIC
carotenoid-associated protein, is up-regulated by GA₃ in a primary fashion. Activation of transcription, post-transcriptional processing, and selective stabilization of the mRNA could all account for the GA₃-enhanced expression. Characterization of the CHRC genomic clone with respect to cis-elements would be of great interest in further delineating the action of GA.

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